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(54) Title: PROCESS AND NUCLEIC ACID CONSTRUCT FOR PRODUCING REAGENT COMPLEXES USEFUL IN DETERMINING TARGET NUCLEOTIDE SEQUENCES		
(57) Abstract <p>A nucleic acid construct useful in preparing reagents for determining target nucleotide sequences in the nucleic acid of a biological sample, the construct having in its single-stranded form: (a) a target binding region substantially complementary to the target nucleotide sequences and (b) a signal strand pairing segment bound in the construct by complementary base pairing to a portion of the target binding region; a second portion of the target binding region being single stranded; and the target binding region and signal strand pairing segment being covalently linked by a phosphate/sugar backbone. A replicable nucleic acid having an origin of replication and two half-restriction sites capable of forming a restriction site can be treated with a restriction enzyme to form a length of nucleic acid containing the target binding region and the signal strand pairing segment. Subsequent labeling of the construct and various optional cleavage and derivation steps can convert the construct to a reagent complex.</p>		

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PROCESS AND NUCLEIC ACID CONSTRUCT FOR PRODUCING
REAGENT COMPLEXES USEFUL IN DETERMINING TARGET
NUCLEOTIDE SEQUENCES

DESCRIPTION

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BACKGROUND OF THE INVENTION

The present invention relates to the production of reagent complexes for the determination of target nucleotide sequences; in particular, novel nucleic acid molecules and constructs are prepared by the process of the present invention which can be replicated and which contain all of the essential nucleic acid components of a reagent complex for a strand displacement assay.

Conventional nucleic acid assays involve a labeled probe polynucleotide. This probe polynucleotide contains a target binding reagent which is complementary to the target nucleotide sequence sought to be assayed. By first immobilizing sample DNA in single stranded form and then probing with the labeled probe polynucleotide, hybridization will occur if and when the target nucleotide sequence is present in the immobilized sample. By washing away unhybridized labeled probe polynucleotide and assaying for label remaining on the surface, a determination can be made whether and how much of the target nucleotide sequence is present in the sample. See U.S. Patent 4,358,535 of Falkow, et al. (1982).

Probe strands complementary to the target nucleotide sequence have been cloned into various vectors for replication before use. Two such types of vectors are viruses having a mature circular single-stranded form (e.g., bacteriophage M13 and bacteriophage F1) and double-stranded circular plasmids (e.g., plasmids pBR322 and PUC) which are replicated in double-stranded form. When such plasmids are provided with origins for replication of a circular single-stranded virus (e.g., the M13 origin of replication), the cells bearing the plasmid having a cloned target binding

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reagent insert which are infected by the bacteriophage will produce virus particles, some of which contain the plasmid-cloned insert DNA in single-stranded form. See A. Levinson et al., J. of Mol. & Appl. Genetics, vol. 2, pp. 507-517 (1984); L. Dente et al., Nucleic Acids Res.,
5 vol. 11, pp. 1645-1655 (1983); R.J. Zagursky and M.L. Berman, Gene, vol. 27, pp. 183-191 (1984).

Strand migration phenomena have been studied. See, e.g., C. Green and C. Tibbetts, Nucleic Acids Research, vol. 9, no. 8, pp. 1905-1918 (1981). The use of strand
10 displacement to assay for the presence and concentration of a target nucleotide sequence is first disclosed in Application U.S.S.N. 607,885 of S.E. Diamond, et al, "Displacement Polynucleotide Assay Method And
15 Polynucleotide Complex Reagent Therefore" (filed May 7, 1984, copending, and assigned jointly to Allied Corporation and Genetics Institute, Inc.) (see EPA 164,876, published December 18, 1985, and EPA 167,238, published January 8, 1986). Reagent complexes for such
20 assays require two polynucleotides: (1) a probe polynucleotide containing the target binding reagent complementary to the target nucleotide sequence to be assayed and (2) a labeled polynucleotide (or signal
25 strand) containing a pairing segment complementary to at least a portion of the target binding region of the probe polynucleotide. While each of these polynucleotides may be cloned separately (or one replicated by cloning and the other chemically
30 synthesized), there are significant disadvantages to the necessity of hybridizing the labeled polynucleotide to the probe polynucleotide in the production of reagent complexes for such displacement polynucleotide assays. In particular, one frequently encounters either probe polynucleotide not bearing a hybridized labeled
35 polynucleotide, labeled polynucleotide not bearing a hybridized probe polynucleotide or some of each. While extensive washings and purifications may overcome such incomplete reagent complexes, such treatments are

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desirably avoided. Furthermore, the other isolation and purification techniques describe in application U.S.S.N. 729,501 of P.D. Unger, et al., filed May 2, 1985, assigned to Allied Corporation and co-pending, while substantially reducing the presence of incomplete reagent complexes, nevertheless provide undesired constraints upon the reagent complex and the method for its use.

BRIEF DESCRIPTION OF THE INVENTION

The present invention provides a method and various intermediate structures in the form of nucleic acid constructs which enable the production and replication of novel precursors for reagent complexes useful in nucleic acids strand displacement assays for virtually any target nucleotide sequence. By providing the two polynucleotide portions of the reagent complex in a single replicable structure, and providing a series of easy steps to manufacture operative reagent complexes from the replicable structure, the present invention overcomes the above limitations.

Accordingly, the present invention provides a process of preparing a reagent complex for determining a target nucleotide sequence in the nucleic acid of a biological sample, which process comprises the steps:

(a) producing by replication a continuous nucleic acid molecule predominantly in single-stranded form having:

(i) a target binding region substantially complementary to the target nucleotide sequence, and

(ii) a signal strand pairing segment bound in the construct by complementary base pairing to a portion of the target binding region; a second portion of the target binding region being single-stranded, and the target binding region and signal strand pairing segment being covalently linked by a phosphate/sugar backbone;

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(b) forming a first terminus adjacent to the signal strand pairing segment and a second terminus adjacent to the target binding region; and

(c) attaching a detectable tag.

5 In many cases, the detectable tag is attached to a terminus adjacent to the signal strand pairing segment and the above three-step process further includes a fourth step: . .

10 (d) cleaving the phosphate/sugar backbone covalent linkage of the signal strand pairing segment to the target binding region.

For the four-step process, the forming step (b) (forming the exterior ends) and the cleaving step (d) (forming the interior ends) may be performed in either
15 order or simultaneously. The attaching step (c) normally follows one or both of the forming step (b) and cleaving step (d) and can involve attachment to an interior end, to an exterior end or attachment elsewhere.

20 The present invention further provides a nucleic acid construct useful in preparing reagent complexes for determining target nucleotide sequences in the nucleic acid of a biological sample, the construct comprising:

25 (a) a target binding region substantially complementary to the target nucleotide sequence, and

(b) a signal strand pairing segment bound in the construct by complementary base pairing to a portion of the target binding region;

30 a second portion of the target binding region being single-stranded; and

the target binding region and signal strand pairing segment being covalently linked by a phosphate/sugar backbone.

35 The present invention further provides a replicable continuous nucleic acid molecule having:

(a) a target binding region substantially complementary to a target nucleotide sequence which is

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to be determined, and

(b) a signal strand pairing segment complementary to a portion of the target binding region and oriented in the opposite direction to the complementary bases of the target binding region; whereby the continuous
5 nucleic acid molecule, when isolated from its complementary nucleic acid molecule, can form a double-stranded segment with the signal strand pairing segment bound to a portion of the target binding region by complementary base pairing.

10 It will be appreciated that the replicable nucleic acid molecule described above may be in single- or double-stranded form (i.e., with or isolated from its complementary strand) and may still contain one or more origins of replication. Preferably, the replicable
15 nucleic acid molecule is replicated in a double-stranded form (e.g., a plasmid) bearing origins of replication both for replication in the double-stranded mode by the host cell and replication to form unique single-stranded structures when the host cell is infected with an
20 appropriate virus whose mature form contains single-stranded nucleic acid. Subsequent treatment of the single-stranded form of the replicable continuous nucleic acid molecule of the present invention (see, e.g., Fig. 1D) produces a form of the nucleic acid
25 construct of the present invention (see, e.g., Figure 1E).

DETAILED DESCRIPTION OF THE INVENTION

In this application the following terms are used based on their generally accepted meanings in the field
30 of molecular biology:

Polynucleotide or Polynucleotide Strand refers to a linear polymeric structure of pentose sugars (generally ribose or deoxyribose) linked to each other by 3', 5' -
35 phosphodiester linkages, and linked by carbon-nitrogen bonds at the 1-carbon of the sugar to pendant purine or pyrimidine bases such as, but not limited to, uracil (linked naturally to ribose only as rU), thymine (linked

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naturally to deoxyribose only as dT), cytosine (dC or rC), adenine (dA or rA) and guanine (dG or rG). Polynucleotides thus include strands of deoxyribonucleic acid (DNA) and strands of ribonucleic acid (RNA) or continuous heteropolymers of both types of polynucleotides.

The ends of such Polynucleotide Strands are referred to as the Five Prime (5') ends, where the 5-carbon of the pentose is not linked to another pentose (but may bear hydroxyl, monophosphate or other natural or synthetic moieties), or the Three Prime (3') ends, where the 3-carbon of the pentose is not linked to another pentose (but may similarly bear hydroxyl, monophosphate or other natural or synthetic moieties).

Complementary Base Pairing or Purine/Pyrimidine Base Pairing refers to the hydrogen bonding between opposite bases pendant on two antiparallel Polynucleotide Strands, which is most energetically favorable for natural DNA when dG is opposite dC and dA is opposite dT. Bases other than the five naturally-prevalent ones also have preferential pairing: for example, 5-methylcytosine binds preferentially to guanine. For illustrative purposes, this pairing is shown in many of the Figures by parallel straight lines with apposite strands directed in antiparallel directions (in the 5' to 3' sense). It should be appreciated, however, that the actual geometry of double-standard segment will normally be helical (the well-known double helix) of various pitches, as schematically illustrated in Figure 1D of U.S.S.N. 607,885.

Hybridization is used herein to refer to admixing two Polynucleotides under these conditions conducive to the formation of double-stranded structures, with Complementary Base Pairing causing such double stranded structures to form where complementary sequences or nearly complementary sequences are present.

The present invention will be described, first, in terms of the process of the invention. Thereafter, the

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continuous nucleic acid molecule of the invention, which is present in early steps of the process, will be described further. Next, the nucleic acid construct, which is present in later steps of the process, will be described further. Finally, the reagent complexes
5 (which may be made by the process) and their use will be described. It should be understood that such reagent complexes and methods for their use are, in general, parts of related inventions such as that of U.S.S.N. 607,885, filed May 7, 1984, in which the present
10 applicants are co-inventors (see EPA 164,876 and 167,238). Certain forms of the use will be described by reference to various related inventions assigned to Genetics Institute, Inc., to Allied Corporation or to the two companies jointly.

15 The present invention is also useful in preparing inverse reagent complexes, as described in U.S.S.N. 809,992 of Collins and Dougherty, filed December 16, 1985, copending and commonly assigned. In such inverse reagent complexes, the label is attached to or
20 incorporated into the construct near the target binding region (in the sense of staying with the target binding region during manufacture and hybridization with the sample). If such inverse reagent complexes are immobilized, the point of attachment is generally near
25 or in the pairing segment so that the labeled target binding region can be easily separated from the immobilized pairing segment when displacement has occurred. The disclosure of U.S.S.N. 809,992 is incorporated herein by reference for a further
30 discussion of inverse displacement. Present Examples 13 and 18 (corresponding to Examples 9 and 10 of U.S.S.N. 809,971 and to Examples 1 and 2 of U.S.S.N. 809,992) illustrate the use of embodiments of the present invention to prepare inverse reagent complexes.

35 The starting material for the process of the present invention is a replicable continuous nucleic acid molecule. It may be in the form of a nucleic acid

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strand of a virus (e.g., a bacteriophage), of a plasmid or of other vectors, or of a replicating organism (e.g., a bacterium or yeast). Preferably, the replicable nucleic acid molecule or strand is of a bacteriophage or plasmid and contains one or more origins of replication recognizable by the replication system either naturally present in, or induced by an infecting virus to be in, the host cell. The replicable continuous nucleic acid molecule is normally DNA; forms of the invention wherein the construct may be RNA are described separately below.

The two essential portions of the replicable continuous nucleic acid molecule are a target binding region and a signal strand pairing segment. These two nucleic acid sequences are the precursors for corresponding segments of the reagent complex used in the method of U.S.S.N. 607,885 to determine or detect a target nucleotide sequence in a biological sample. Accordingly, for reasons discussed in U.S.S.N. 607,885, the overall target binding (TBR) may be of any desired length to impart sufficient specificity, but is commonly about 35 to about 2000 nucleotides in length, and is preferably about 100 to about 1000 nucleotides in length. The manufacturing process of the present invention imposes no substantial limitations upon the choice or size of target binding region.

The signal strand pairing segment (PS) is the precursor for the portion of the labeled polynucleotide (L in U.S.S.N. 607,885), called herein the signal strand, that is bound by complementary base pairing to a portion (LBR) of TBR in the reagent complex. As indicated in U.S.S.N. 607,885, the length of LBR can be, for example, 20 to 1000 nucleotides, and is preferably 50 to 500 nucleotides and is more preferably 100 to 300 nucleotides. It will be appreciated that a target binding region larger than LBR leaves one or more initial binding regions (IBR) as discussed in U.S.S.N. 607,885. The pairing segment (PS) has a corresponding length to LBR, but contains the complementary bases

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(except for any permissible mismatches) and is in inverse order within the replicable nucleic acid molecule. As described in more detail below, the inverse order of complementary bases in PS compared to LBR (part of TBR) permits the continuous nucleic acid molecule, once in single-stranded form (isolated from its complementary strand) to form a double-stranded region with PS bound to LBR. As indicated in U.S.S.N. 607,885, some mismatched bases may exist in the region of complementarity. As illustrated by Figure 1D (and described later), there may be other regions of local double-strandedness besides LBR-PS, and the replicable nucleic acid molecule may still be intact. While this inverted repeat structure is normally able to form upon isolation from the complementary strand, it is contemplated that such formation may, in some cases, be aided by linearization of the nucleic acid molecule or by other biochemical, chemical or physicochemical steps such as selected conditions of temperature or ionic strength.

In certain preferred forms of the continuous nucleic acid molecule, a restriction site recognizable by and cleavable by a restriction endonuclease, is also formed in the continuous nucleic acid molecule isolated from its complementary strand (see the Bam HI site in Figure 1D). Cleaving that site cuts the continuous nucleic acid molecule at two points. By locating those two points on the exterior of a segment including and connecting TBR and PS covalently (by the phosphate sugar backbone), a length of nucleic acid can be isolated from the remainder of the replicable nucleic acid molecule. Alternatively, the two points can be restriction sites created by separate inverted repeats (as is segment 16 in the Figures). This length either has the LBR/PS double-stranded segment or can form such segment. There is no critical maximum on the nucleotides not part of TBR or of PS. This length of nucleic acid, containing both TBR and PS, and having PS bound by base pairing to

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a portion (LBR) of TBR is called herein the nucleic acid construct.

As discussed below, the nucleic acid construct may be RNA transcribed from a DNA nucleic acid molecule, either directly or after cutting a long DNA molecule
5 which contains an origin of transcription.

As formed, the nucleic acid construct has two ends (a 5 Prime End and a 3 Prime End), one of which is exterior of TBR along the nucleic acid chain, the other
10 of which is exterior of PS along the nucleic acid chain. As described more fully below, each of these two ends may be used in preparing reagent complexes or, alternatively, one of these ends and one new end created by cutting between TBR and PS may be used. While the
15 additional cut will create two new ends (a second 5' Prime End and a second 3' Prime End), normally, at least one of the initially-created (exterior) ends is used.

While much of the present description is of a process wherein the exterior ends are created first (to produce the nucleic acid construct) and then the
20 interior cut (if any) is made, such order may be reversed or the two sets of cuts may be made concurrently or without any intervening purification or separation. Furthermore, as discussed at the end of
25 Example 7, the continuous nucleic acid molecule can be labeled and used in a displacement/capture assay without creating either exterior or interior ends.

At some point, for the preparation of ordinary reagent complexes to be used in the method of U.S.S.N. 607,885, a detectable tag is attached to an end adjacent
30 to the signal strand pairing segment. For inverse reagent complexes (see U.S.S.N. 809,992), the tag is normally attached to an end adjacent to the target binding region. The term "adjacent to" in this context means sufficiently close, as measured topologically
35 along the polynucleotide chain (as distinct from spatially) that the tag will remain with the signal strand pairing segment (or, for inverse reagent

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complexes, with the target binding region) throughout the process of preparing reagent complexes, and will remain with the signal strand (or, for inverse reagent complexes with the target binding region) if it is displaced from TBR (or from PS) by a target nucleotide
5 sequence. The end to which the detectable tag is attached may be an interior end or an exterior end, and may be a 5 Prime end or a 3 Prime end. As described below and in U.S.S.N. 809,992, if the entire ordinary or
10 inverse reagent complex is to be captured after displacement, there are suitable forms of the invention wherein the tag is attached randomly at one or more sites on the molecule, e.g., by photochemical or non-specific chemical reaction (see Example 9).

Suitable detectable tags include those described in
15 U.S.S.N. 607,885, including radioisotopes, fluorescent molecules, enzymes and chemiluminescent tags. Other suitable detectable tags are lengths of ribonucleotides and especially of poly (riboadenosine) as described in C. Vary, et al., U.S.S.N. 729,503, filed May 2, 1985,
20 assigned to Allied Corporation and copending. At the 3 Prime End, various such tags can be incorporated by chain elongation, especially with terminal deoxynucleotidyl transferase (TdT). Elongation with
25 ribonucleotides and especially riboadenosine may involve the enzyme polynucleotide phosphorylase (PNP), as described in that application.

Detectable tags may also be attached by chemical modification, by ligation using splint oligonucleotide or by a combination of ligation and chemical
30 modification as described in E. Brown, et al., U.S.S.N. 729,700, filed May 2, 1985, commonly-assigned, and copending. Such techniques may be specific for any 5 Prime end, for any 3 Prime end or (especially when ligation is involved) for 5 Prime or 3 Prime ends having
35 specified sequences.

In some forms of the invention applied to the reagent complexes of U.S.S.N. 607,885, an end (interior

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or exterior) adjacent to the target binding region (TBR) is also used. Normally, such end is either attached to a solid phase to create an immobilized probe polynucleotide (as described in U.S.S.N. 607,885), or is
5 attached to a moiety (e.g., biotin), that can be selectively attached to a solid support at a later time (normally after the assay method). Such use of an immobilizable probe is described in U.S.S.N. 607,885. Additional forms of such immobilizable probe are
10 described in Unger, et al., U.S.S.N. 729,501, copending, assigned to Allied Corporation and filed May 2, 1985. In similar fashion, for preparing certain forms of the inverse reagent complexes of U.S.S.N. 809,992, an end (interior or exterior) adjacent to the pairing segment (PS) can be either attached to a solid phase
15 (immobilized) or rendered immobilizable. Techniques for rendering such end immobilizable include elongation with biotinylated dUTP, elongation with dCTP (to form poly-dC) or attachment chemically of biotin or some other affinity moiety.

20 The reagent complexes made in accordance with the present invention can be used for the detection and determination of a variety of target nucleotide sequences in a variety of concentrations. In particular, microorganisms including infectious agents
25 whose nucleic acid (genomic or otherwise) could be targeted include pathogenic viruses, bacteria and fungi; e.g., cytomegalovirus or Neisseria gonorrhea. Exemplary genetic disorders or conditions which could be targeted include β thalassemias, α_1 -thalassemias, cri du chat
30 syndrome and some retinoblastomas. The methods and reagents of U.S.S.N. 607,885 are applicable to detecting genetic disorders or variations primarily when a multi-base nucleotide deletion, insertion, substitution or
35 transposition is involved in distinguishing the target sequence. To the extent that such methods are applicable to genetic disorders due to single base mutations, if at all, the complement of the substituted

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base or other point of mutation is desirably part of the target binding region of the probe polynucleotide, with the location of that base within the region likely to affect the selectivity of the method. Among changes in structural or regulatory genes, changes or differences
5 in the expression, activation or rearrangement of oncogenes can be detected by the present process. Other perturbations in the expression of structural genes can be similarly detected. Such reagent complexes can also be applied to HLA typing for tissue transplantation,
10 determination of antibiotic resistance genes in microorganisms, and to the screening of food, medicinal and water samples for specific infectious agents or other microorganisms.

15 Selecting a target sequence for a particular test may involve determining a sequence which is unique or relatively unique to the target organism or condition. Such target sequences would be used to develop the target binding region as complementary thereto, and then a pairing segment for the signal strand polynucleotide
20 of appropriate length would be developed to bind to a part of the target binding region. Such segments can then be inserted into a cloning vector as described below.

25 Figure 1A illustrates one embodiment of a double-stranded nucleic acid 10, the top strand of which forms a part of the replicable nucleic acid molecule of the present invention. The top strand of segment 10 has, from left to right (being the 5' to 3' direction), a first segment 12, an intervening region with an Sma I
30 restriction site, a second segment 16, an intervening region with an Xba I restriction site, an Hinc II restriction site and a Pst I restriction site and a third segment 14 at the 3' end of the top strand of nucleic acid 10. The bottom strand, in antiparallel
35 direction, is fully complementary and has from right to left (its 5' to 3' direction): a segment 14' of the bottom strand complementary to the third segment 14, an

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intervening region, a segment 16' of the bottom strand complementary to second segment 16, an intervening region and a segment 12' of the bottom strand complementary to first segment 12. The intervening regions, being fully complementary, complete, respectively, the Sma I restriction site between the 12/12' region and the 16/16' region and the Xba I, Hinc II and Pst I restriction sites between the segment 16/16' region and the 14/14' region.

Nucleic acid 10 may be part of any of a variety of double-stranded nucleic acids, including genomic DNA of a microorganism of various cells, and including plasmids, viruses and other vectors capable of replication in double-stranded form in a host cell.

Figure 1B illustrate the top strand of nucleic acid 10 shown in Figure 1A in continuous (primarily single-stranded) form. As explained in the discussion above, the single-stranded form may be produced by physico-chemical manipulation or may represent the native form of a virus such as M13 (which is in double-stranded form during replication). Preferably, the single-stranded form is the result of incorporation of one strand of a normally double-stranded polynucleotide into virus particles (e.g., by M13 infection of a microorganism containing a double-stranded plasmid DNA having an M13 origin of replication).

In Figure 1B, the precise sequence of the single (top) strand is shown, with various restriction sites and half-restriction sites also shown. The term "restriction site" is meant to indicate a double-stranded region having a sequence recognizable by and cleavable by a specific restriction endonuclease. The term half-restriction site is meant to indicate a single-stranded nucleotide segment which, when it is hybridized to the perfectly matched nucleotide segment, forms such a restriction site. Because segments 12 and 14 of this top strand from Figure 1A are complementary to each other, they form a 22 base pair duplex segment

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having a Bam HI restriction site at bases 10-15 of segment 12 (counting from the 5' end of segment 12) and at the corresponding bases of segment 14. Segment 16 is self-complementary, such that this 52 nucleotide segment forms a 26 base pair duplex hairpin structure, with an
5 Eco RI restriction site at bases 9-14 and 39-44 of segment 16. The first intervening sequence (with an Sma I half-restriction site) and the second intervening sequence (with Xba I, Hinc II and Pst I half restriction sites) each join the segment 12/14 duplex to the segment
10 16 duplex hairpin structure with short single-stranded nucleotide sequences.

In Figure 1C insertion of two double-stranded nucleotides into nucleic acid 10 is shown. At the Sma I site (see Figure 1A) a double-stranded polynucleotide
15 TBR is inserted having a left portion (LBR) and a right portion (IBR). As indicated below, the combination of LBR and IBR will be chosen such that a defined segment (TBR) of one strand is complementary to a target
20 nucleotide sequence that is desired to be determined or detected. The TBR insert is shown here as having LBR on the exterior (left) side and IBR in the interior (right) side. At the Pst I site (see Figure 1A) a segment PS is inserted that is substantially homologous (in an inverse
25 orientation) to the LBR portion of the first insert TBR.

The generality of the present invention can be appreciated by considering that any sequence of virtually any length can be synthesized, cloned or otherwise obtained and inserted in the desired
30 orientation as the first insert (at the Sma I site) for use as the target binding region TBR of the probe polynucleotide. Thereafter, any convenient portion of the first insert can be chosen and its complementary sequence either chemically synthesized, replicated or
35 both to serve as the second insert PS (which will ultimately serve as the pairing segment of the signal strand or labeled polynucleotide).

Figure 1D shows a complete circular single-stranded

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DNA molecule (the replicable nucleic acid molecule of the invention in single-stranded form) containing the top strand from the product of insertion as illustrated in Figure 1C. It has been isolated from its complementary strand (which contains segments 10', 12', 14' and 16'). Looking in the direction from the 5' end to the 3' end of each segment, the main loop of the circular DNA is connected by segment 12 and a short single-stranded segment to segment LBR, then segment IBR, then the hairpin 16, then a single-stranded segment containing half restriction sites Xba I and Hinc II, then segment PS (hybridized to segment LBR), then a short single-stranded sequence, then segment 14 (hybridized to segment 12) and finally back to the other (5') end of the main loop. The main loop contains a segment CE which, when it is in double-stranded form, could serve as an origin for replication in double-stranded form (col EI origin in the embodiment of the Examples). The main loop also has an origin for M13 replication (shown as M13 ori).

Cleavage of the circular, primarily single-stranded DNA shown in Figure 1D by the restriction endonuclease Bam HI (at a Bam HI site within segment 12/14, see Figure 1B) produces the loop shown in Figure 1E with only short pieces of DNA to the left of the segment LBR/segment PS duplex. Depending upon temperature and salt concentration, these pieces may be single-stranded or joined at the end by a 12 based-paired duplex. In particular, the 5' end structure is 5'-GATCCGTCGACCCGCC-LBR; the 3' end structure is 3'-GCAGCTGGTCGAACCCG-PS; and the eight underlined nucleotides can be base-paired. The GATC overhang at the 5'-end is referred to below.

At this point, a second nucleic acid construct has been formed which includes:

(a) a target binding region (segments LBR and IBR) substantially complementary to the target nucleotide sequence, and

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(b) a signal strand binding region (segment PS) bound in the construct to a portion (LBR) of the target binding region,

a second portion (IBR) of the target binding region being single stranded, the target binding region and signal strand binding region being covalently linked by a phosphate/sugar backbone (the segment joining PS to IBR which includes hairpin 16 and half-restriction sites Xba I and Hinc II). Such construct may be separated from the larger DNA fragment, e.g., by hydroxyapatite chromatography.

The nucleic acid construct is now susceptible to a variety of the specific chemical or biochemical modifications at either the existing 3' end, the existing 5' end or at 3' and 5' ends that will be created when the Eco RI restriction site of hairpin 16 is cleaved. A first example of such series of modifications is illustrated in Figures 1F and 1G.

Starting with the nucleic acid construct of Figure 1E, the 3' end (near segment PS) can be modified to attach a detectable tag T as shown in Figure 1F. Purely chemical modifications may be used such as described in U.S.S.N. 607,885. Biochemical modifications that may be made specifically at this 3' end include those based either upon filling in of the bases CTAG complementary to the GATC overhang left in single-stranded form by the action of Bam HI restriction endonuclease (see above sequence of the 5' end), or by elongation at the 3' end by the action of various enzymes such as terminal deoxynucleotidyl transferase.

In such fashion radioactively labeled nucleotides may be added to the 3' end. Nucleotides such as biotinylated uridine may also be added to the 3' end by elongation with deoxynucleotidyl terminal transferase; such elongation may be followed by binding streptavidin-enzyme conjugates to the pendant biotins. Alternatively, a series of riboadenosine nucleotides may be added by the combined action of deoxynucleotidyl

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terminal transferase followed by polynucleotide phosphorylase as described in application U.S.S.N. 729,503 of C. Vary, et al. entitled "Diagnostic Reagent, Kit And Method Employing Polynucleotide Displacement, Separation, Enzymatic Cleavage And Adenosine Phosphate Detection," and in U.S.S.N. 729,502, also filed May 2, 1985.

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Whichever tag is chosen, the tagged construct of Figure 1F may now be attached to a solid phase specifically by the free 5' end as illustrated in Figure 1G. Looking at the top portion of the Figure 1G, a DNA attachment moiety AM is covalently attached to a solid phase and a splint oligonucleotide SP is used to hybridize both to the 5' end of the tagged second construct and the 3' end of the attachment moiety AM. Ligation forms a complete covalent attachment of the tagged construct to the solid phase. The steps for this attachment may be presented in several orders, with one preferred order being to: (1) hybridize attachment moiety, splint and nucleic acid construct, (2) ligate to form a covalent link between the attachment moiety and the free 5' end of the construct adjacent to TBR, and (3) chemical attachment of the other end of the attachment moiety to the support. See E. Brown et al., U.S.S.N. 729,700. By then digesting with restriction endonuclease Eco RI, the construct can be cut at the hairpin 16, causing the probe polynucleotide P (containing segments LBR and IBR) to cease to be covalently linked to a labeled polynucleotide L (as in Figure 1G) This severing may occur before or after attachment to the support. The labeled polynucleotides contain segment PS and the half-restriction sites Hinc II and Xba I, as well as the tag T. The labeled polynucleotide L remains bound to the probe polynucleotide P via the purine/pyrimidine hydrogen bonding between segment PS and segment LBR.

As described more in fully in application U.S. Serial No. 607,885, this reagent complex may now be used

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to assay for a target nucleotide sequence complementary to segments LBR and IBR. A sample, if it contains the target nucleotide sequence, will hybridize first at the single-stranded portion IBR of the probe polynucleotide P (the right end of probe P in Figure 1G), forming a duplex structure, and thereafter, branch migration will occur toward the left in Figure 1G. While the branching point may shift back and forth to the left and right, especially within segment LBR which is complementary both to the pairing segment PS of the labeled polynucleotide L and a portion of the target nucleotide sequence, eventually the branch will shift sufficiently to the left that the labeled polynucleotide L will dissociate from the probe polynucleotide P. Under normal conditions, such displacement of the labeled polynucleotide L can occur quickly after the beginning of hybridization of the target nucleotide sequence to the initial binding region IBR (typically within 30 minutes of total assay time and, on a microscopic level, within seconds of initial hybridization at segment IBR).

By separating the liquid phase containing displaced labeled polynucleotides L from the solid phase containing attached intact reagent complexes, and then assaying for the tag T in either phase (but preferably in the liquid phase), a measurement can be made which is both qualitatively and quantitatively a function of the presence and the concentration of the target nucleotide sequence in the sample. Such method of use is described in greater detail in U.S.S.N. 607,885.

Figure 1H shows an immobilizable reagent complex that can be prepared from the tagged construct of Figure 1F. By digesting the tagged construct of Figure 1F with restriction endonuclease Eco RI, the hairpin 16 is cleaved, removing the covalent attachment of the probe polynucleotide to the labeled polynucleotide L. A second 3' end is now created near the 3' end of segment IBR. Assuming that the tag T is one which blocks chain elongation, the action of terminal deoxynucleotidyl

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transferase in the presence of biotinylated uridine (see EPA 63,879 (1982)) will attach a series of uridine nucleotides with pendant biotins as shown on modified probe P' in Figure 1H. Such pendant biotins may now serve as a site for attachment by immobilized avidin or streptavidin (shown as A's on a solid phase). As indicated in U.S.S.N. 607,885, such affinity attachment may either serve as part of the preparation of an immobilized reagent complex or serve as a means of separation following a specific displacement assay run in solution.

In many forms of the invention, two internal half-restriction sites between segment TBR and segment PS within the construct can pair to form a restriction site (hairpin 16 having an Eco RI site in Figures 1D and 1E) which is cut at some point in the process. Other forms of recognizable-cleavage sites could be used in place of such hairpin. There are many other forms of the invention, however, in which such a site need not be present or, if present, that it need not be cut during the process, provided that some means is present for distinguishing: (1) segments PS bound by complementary base pairing to segments LBR from (2) segments PS unbound by complementary base pairing (displaced from) segment LBR. As indicated in Figure 3C of U.S.S.N. 607,885 and the accompanying description, a second tag may be present on the probe polynucleotide which is in spatial proximity to the tag on the labeled polynucleotide when PS is bound to LBR. Upon displacement of PS from LBR, the two tags become separated spatially, even though still attached by the nucleotide chain. If, for example, one tag is a fluorescer and the other tag is a quencher, then the fluorescent signal can be detected selectively from constructs which have been subjected to displacement by target nucleotide strand. Referring to present Figure 1E, such a construct can be formed by attaching the fluorescer to the (exterior) 3' end adjacent to LBR and

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by attaching the quencher to the (exterior) 5' end adjacent to PS.

Figure 2A illustrates a nucleic acid precursor 110 of double stranded nucleic acid. The region of DNA shown in Figure 1A comprises a sequence having segments 12, 16 and 14 and the half-restriction sites Sma I, Xba I, Hinc II and Pst I, which have been placed in nucleic acid 110 in a vector (which includes the M13 and plasmid origins of replication and a selectable marker) in an orientation opposite to that of Figure 1A.

Thus, in Figure 2A, the top strand contains from left to right (the 5' to 3' direction): segment 14', the Pst I, Hinc II and Xba I half-restriction sites, segment 16', the Sma I half-restriction site and segment 12'. Figure 2B illustrates the top strand of nucleic acid precursor 110 shown in Figure 2A in continuous (primarily single-stranded) form, and thus corresponds to the view of Figure 1B.

Figure 2B shows that insertion of the segment in the opposite orientation places segments 14', 16' and 12' in the top strand, which is the strand found in mature virus particles. Therefore, the Sma I site is now near the 3' end that will be created when segment 12' is cleaved; and the Pst I site is now near the 5' end that will be created when segment 14' is cleaved.

Figure 2C illustrates a second embodiment of the nucleic acid construct of the present invention that can be prepared from the precursor shown in Figures 2A and 2B. Figure 2C is analogous to Figure 1E in that the TBR segment (including LBR and IBR) has been inserted at the Sma I site and the PS segment inserted at the Pst I site and the resulting circular single-stranded DNA has been cleaved with restriction endonuclease Bam HI. This construct now contains a free 3' end adjacent segment LBR and contains a free 5' end adjacent segment PS. Attachment of the tag specifically at the 5' end can proceed via a variety of techniques: as described below polynucleotide kinase can replace the terminal 5'

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phosphate with a P-32 labeled phosphate. Furthermore, chemical attachment schemes can be used to attach a variety of detectable tags including fluorescent, enzymatic and chemiluminescent tags specifically to the 5' end. Furthermore, ligation or a combination of ligation and chemical attachment may be used, as described in U.S.S.N. 729,700 of E. Brown, et al, referenced above.

Once the tagged (at the 5' end) nucleic acid construct is formed, it may then be digested with the restriction endonuclease Eco RI. This enzyme cleaves the hairpin 16, forming the linear structure shown on the left side of Figure 2D. In this linear structure, the labeled polynucleotide L contains the pairing segment PS and at its 5' end the detectable tag T. The longer polynucleotide, probe polynucleotide P, contains segments LBR and IBR. While the polynucleotides L and P are no longer covalently joined, they are joined by hydrogen bonding between segments PS and LBR. Furthermore, while both polynucleotide L and P have free 3' ends, only the probe polynucleotide P has a free 5' end.

Referring to the remainder of Figure 2D, the 5' end of the probe polynucleotide P of the reagent complex may now be attached to a solid surface SU by an attachment moiety AM and a splint SP (followed by ligation) as described in more detail in application U.S.S.N. 729,700 of E. Brown, et al. Thereafter, the immobilized reagent complex may be used for a displacement assay as described in U.S. Application Serial No. 607,887 of S.E. Diamond, et al. Alternatively, rather than attaching the 5' end of the probe polynucleotide P of the reagent complex of Figure 2C to a support, a moiety B may be attached at the 5' end of the probe polynucleotide P (such as biotin or iminobiotin via ligation to a second attachment moiety containing biotin or iminobiotin) so that the displacement reaction may be run in solution. After completion of displacement reaction, the attached moiety at the 5' end of the probe P can be used to trap

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with an immobilized affinity reagent (e.g., streptavidin) all of the intact reagent complexes. Under these circumstances, only displaced labeled polynucleotides L (no longer bound to probe polynucleotide P) should pass through the immobilized affinity material for detection. Again, the quantity of tag detected in the eluant from a column of immobilized affinity reagent will be functionally related to the presence and concentration of target nucleotides sequence present in the sample used for the displacement.

It should be appreciated that a replicable nucleic acid molecule and embodiments of the nucleic acid construct having similar 5' ends adjacent to PS and similar 3' ends adjacent to TBR can also be produced by reversing the insertion shown in Figure 1C into the precursor strand 10 shown in Figure 1A. One would thus introduce segment PS at the Sma I site (using appropriate end nucleotides) and introduce segment TBR at the Pst I restriction site (using appropriate end nucleotides). Thus, the specificity of which segment (TBR or PS) is adjacent to a free (exterior) 5' or 3' end can be determined on the basis of which precursor replicable nucleic acid molecule is used (molecule 10 of Figure 1A or molecule 110 of Figure 2A) and which sites (Sma I or Pst I) the TBR or PS are inserted at. It should be appreciated that the Sma I and Pst I sites are specified as such for illustrative purposes based upon the precursor nucleic acid molecule of Example 4. One skilled in the art could find other half-restriction sites or other techniques to specifically place or clone segments TBR and PS in the desired location and orientation.

Inverse reagent complexes can similarly be constructed from the constructs either of Figure 1E or of Figure 2C, with the attachment of a tag now being directed specifically to the end (the 5' End in Figure 1E; the 3' End in Figure 2C) adjacent to the target

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binding region TBR (IBR plus LBR). If one desires to then immobilize the pairing segment PS or render it immobilizable, appropriate chemical or biochemical modification can then occur at the other exterior end (the 3' End in Figure 1E; the 5' End in Figure 2C) or, after cleaving the hairpin, at the interior end formed adjacent to the pairing segment PS.

Figure 3A, 3C and 3E illustrate three modifications of the nucleic acid construct of Figure 1E. Each of these modified forms can be produced by methods similar to those shown in Figure 1C, but using pairing segments PS complementary to different portions of the first insert TBR. Thus, in Figure 3A, a pairing segment PS has been chosen for insertion at the Pst I site (see Figure 1A) which is complementary to the second fifth of the target binding region TBR. Under these conditions, the second construct of Figure 3A contains a significantly longer (compared to the second construct of Figure 1E) single-stranded portion near the 5' end, which portion now contains the 5' end of the target binding region (IBR-1). Dissociation of the relatively short duplex region at the ends of the nucleotide strand will now create a significantly longer 5' end for elongation analogous to that illustrated in Figure 1H. Furthermore, the labeled polynucleotide L will now be present nearer to the middle of the target binding region TBR in the immobilized reagent complex, shown in Figure 3B, that can be produced (compare Figure 1G).

Figure 3C illustrates a nucleic-acid construct, similar to that shown in Figure 1E, but now with pairing segment PS complementary to a labeled polynucleotide binding region LBR near the middle of the target binding region TBR. This shift increases the size of the 5' single-stranded region (including IBR-3) of the second construct and decreases the looped portion (including IBR-4) between segment LBR and the hairpin 16. Figure 3D shows a reagent complex that can be prepared therefrom.

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Figure 3E illustrates a nucleic acid construct similar to that illustrated in Figure 1E, but now with the pairing segment PS complementary to a segment LBR at the 3' end of the target binding region TBR. Now all of the initial binding region IBR is closer to the 5' end than the labeled binding region LBR. Therefore the hairpin 16 forms the major portion of the loop connecting the duplex PS/LBR. Once a tag is attached to the 3' end and the 5' end is immobilized, (followed by cutting the hairpin Eco RI restriction site), a reagent complex will be created in which the labeled polynucleotide L is hybridized to the distal end of the probe polynucleotide P relative to the solid phase to which the 5' end of the probe polynucleotide is attached.

Referring again to Figures 1D and 1E, one can also provide for different lengths of intervening sequences, especially between segment 12 and segment LBR. Additional nucleotides inserted there, either as a part of the precursor nucleic acid or along with LBR and TBR in the insert, will lengthen the spacing in Figure 1F between the exterior 5' end and segment LBR. Such lengthening would (like the constructs of Figures 3A, 3C, and 3E) increase the spacing between a 5' end attachment (e.g., to the support as in Figure 1G) without splitting IBR or placing any of IBR closer to the 5' end than LBR.

Exemplary constructs formed from RNA may be also made using, for example, the SP6 vectors of D.A. Melton, et al., Nucleic Acids Res., vol. 12, pp. 7035-7056 (1984). These vectors, commercially available from Promega Biotec, are derived from pUC12 and contain an SP6 transcription promoter and a polylinker next to the promoter. Fragments of interest, having segments analogous to TBR and PS, would be cloned into different sites in the polylinker region, in an inverted orientation with respect to each other. In lieu of segment 16, a short unique segment would be present

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between the TBR and PS inserts, such segment being provided by additional nucleotides in the polylinker, or by a third insert in between TBR and PS. In addition, a restriction enzyme site, which is not present in any of the inserts, is present at the end of the inserted
5 region farthest from the SP6 promoter.

In use, the plasmid DNA would be prepared and digested with the restriction enzyme at the end of the insert region to linearize the plasmid. Full-length RNA
10 would be transcribed from the linearized plasmid using purified SP6 polymerase. DNA would be removed with (RNase-free) DNase digestion, and the RNA purified by phenol/chloroform extraction and ethanol precipitation or Sephadex Column Chromatography. Under optimal
15 conditions, up to 10 ug of RNA can be obtained from 1 ug of plasmid in a 50 ul reaction mixture.

The product of this reaction would be an RNA molecule with a duplex region formed by intramolecular hybridization of PS to the LBR region of TBR. TBR and PS would be connected by the single stranded RNA
20 segment. DNA oligomer (typically 6 or more nucleotides in length) complementary to this connecting segment would be provided after transcription. By hybridizing with DNA oligomer and digesting the DNA/RNA hybrid specifically with RNase H (see H. Donis-Keller, Nucleic
25 Acids Res., vol. 7, pp. 179-192 (1979)), specific cleavage of the loop connecting TBR to PS could be accomplished.

In effect, the manufacture of RNA constructs involves steps analogous to Figures 1A-1E with the DNA
30 counterpart, but with the segment defined by Bam HI cleavage in the DNA counterpart being defined by transcription of the region between the SP6 promoter and the restriction enzyme cleavage site distal to the inserted region (for example, the Eco RI site when SP64
35 constructs are used, by Hind III when SP65 constructs are used). Forming the RNA/RNA hybrid segments could be performed simultaneously with forming the DNA

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oligomer/RNA hybrid (analogous to segment 16). Labeling could follow or precede such hybrids being formed and/or cleavage with RNase H. Attachment and/or labeling could be performed using the techniques of E. Brown, et al., U.S.S.N. 729,700.

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Example 1 - Preparation of Nucleic Acid Molecule
Containing Target Binding Region For Albumin Gene And
Pairing Segment

This example describes the preparation of a replicable, continuous nucleic acid molecule in double-stranded form (i.e., with its complementary strand) containing a target binding region for 500 bases of a human albumin gene. The starting materials were E. Coli plasmids, each containing an M13 origin of replication, designated pMLC12 and pMLC13. These two plasmids were, in turn, prepared by partial Hind III digestion and Klenow end fill of the plasmids pSDL12 and pSDL13 (see Figure 5 and accompanying description of A. Levinson et al., J. Mol. & Appl. Gen., vol. 2, pp. 507-517 (1984).

A) Preparation of pMLC12/13Δ M7 (M13 ori plasmid with Mp7 polylinker)

pMLC12 and pMLC13 were digested with EcoRI to completion. The large fragment from pMLC12 (Fragment A) and the small fragment from pMLC13 (Fragment B) were combined, ligated and transformed into MC1061 (F⁻;hsdR, Δ ara-leu 7697, araD139, Δ lac X 74, galU, galK, rpsL (str^r) Casadaban Y. Cohen, J. Mol. Biol. vol 138 pp. 179-207. Chloramphenicol resistant colonies were picked and the correct plasmid (pMLC12/13Δ) was identified on the basis of loss of the BamHI cleavage site seen in both the original pMLC12 and pMLC13 plasmids.

pMLC12/13 Δ was digested partially with EcoRI by use of limited amounts of EcoRI so that only one of the two EcoRI sites is digested in most molecules. The partially cut, linearized pMLC12/13Δ was isolated following gel electrophoresis. Mp7 DNA was digested with PvuII and a 383 bp fragment was isolated following gel electrophoresis. The PvuII fragment was digested with EcoRI to produce the 52 bp EcoRI fragment containing the Mp7 polylinker and two other PuvII/EcoRI fragments (about 123 bp and about 208 bp). The EcoRI digested PuvII fragment from Mp7 and the linearized,

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partially EcoRI digested pMLC12/13A were ligated transformed into MC1061 and chloramphenicol resistant cells were selected. Individual colonies were then grown and DNA was prepared. Plasmids which had correctly incorporated the EcoRI polylinker from Mp7
5 were identified by the acquisition of a BamHI site. The correct plasmid, termed pMLC12/13A M7, is shown in Figure 4A.

10 B) Construction of Albumin-inverted repeat in pMLC12
pMLC12 was digested to completion with HincII. The plasmid pAlbB6 which contains a portion of a human albumin cDNA clone (see Lawn et al., 1980) was digested to completion with PvuII and HincII. The fragment which we wish to clone is the 915 bp PuvII/HincII fragment.
15 The pMLC12/HincII vector and pAlbB6/PuvII + HincII were ligated together and transformed into MC1061. Clones which had incorporated the correct albumin fragment were identified by colony hybridization (Grunstein and Hogness, 1976) using the alb 32mer
20 (5'ACATCCTTGCCTCAGCATAGTTTTTGCAAAC3') as a hybridization probe. Positive colonies were picked and grown up; and DNA from individual colonies was digested with HindIII + PstI to determine the orientation of the inserts. Two plasmids with the albumin fragment insert
25 into the vector in opposite orientations (F31a and F31c) were selected for further steps. Plasmids F31a and F31c are shown in Figure 4B.

F31a was digested to completion with EcoRI + BamHI and the two large fragments were isolated. F31c was
30 digested to completion with EcoRI + BglII and the small (approximately 550 bp) fragment (between the RI and Bgl II sites) was gel isolated. The gel isolated fragments from F31a and F31c were ligated together and transformed into DH1 (ATCC #33849) a *recA*⁻ bacterium. (Note: A
35 *recA*⁻ host was used at this point to reduce the possibility of deletion of one or both copies of the inverted repeat through a *recA* mediated mechanism.

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Subsequent experiments have shown that these inverted repeat clones are stable even in the absence of *recA*⁻ mutation.) DNA was then prepared from individual chloramphenicol resistant colonies and digested separately with PstI, EcoRI plus HindIII, or BglII plus Bam HI to identify clones with the correct structure. One such clone F41a (shown in Figure 4B) was used for further analysis.

F41a was digested with EcoRI and HindIII and blunted with the klenow fragment of DNA Polymerase I. The approximately 1500 bp fragment was isolated following gel electrophoresis. pMLC 12/13Δ M7 was digested to completion with Acc I and blunted with the klenow fragment of DNA Polymerase I. The pMLC12/13Δ M7/AccI + klenow [fragment] and the gel-isolated fragment from F41a were joined by DNA ligase and transformed into MC1061. Plasmids which had incorporated the gel isolated fragment were identified by hybridization to the albumin 32 mer and were verified by digestion with PstI, BamHI, or XbaI. This plasmid, termed pMLC12/13ΔM71VR, is shown in Figure 4C.

For the next step, plasmid MpTL poly was used. This plasmid had been previously prepared by phosphorylating the lower oligonucleotide strand of:

25 EcoRI
5'-HO-TCACGAATTCATCTGTCAAGG-OH-3'
3'-HO-GTGCTTAAGGTAGACAGTTCC-OH-5'

After ligating, a dimer was isolated having the middle portion:

30 STU I
-ATCTGTCAAGGCCTTGACAGAT-
-TAGACAGTTCCGGAAGTGTCTA-

recognizable because of the Stu I site that had formed. The dimer was filled with Klenow fragment of DNA polymerase I so as to have the end portions:

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Eco RI	Eco RI
5' TCACGAATTCC-	-GGAATTCGTGA-3'
3' AGTGCTTAAGG-	-CCTTAAGCACT-5'

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with the underlined base having been filled in by Klenow. This duplex was now cloned into Mp8/Hinc II and a clone picked on the basis of acquisition of a Stu I site. After sequencing for confirmation, the resultant plasmid was MpTL poly.

5 pMLC12/13ΔM71VR was partially digested with XbaI (which cuts four times within the plasmid), blunted as above, and full length linearized plasmid DNA was isolated by gel electrophoresis. The plasmid MpTL poly was digested to completion with BamHI + HindIII and was 10 blunted as above. The 80 bp blunted fragment was isolated by gel electrophoresis and ligated to the gel isolated, linearized, XbaI partially cut, blunted pMLC12/13ΔM71VR. The DNA was transformed into MC1061 and screened with the oligonucleotide which is 15 complimentary to the TL polylinker. Positive colonies were picked and plasmid which had incorporated the TL polylinker at the correct XbaI site was identified by digestion with StuI + Bgl II. This plasmid, termed pMLC12/13Δ M71VRTL, is shown in Figure 4D, and is 20 analogous to Figure 1C.

Two other constructions could be carried out as for the one shown in Figure 4D, above, except that the TL polylinker plasmid would be replaced by:

25 a) a HincII/StuI fragment from the human erythropoietin cDNA (Jacobs et. al., Nature, 1985) which contains two HaeIII sites (which will digest in single-stranded DNA; the albumin cDNA has no HaeIII sites).

30 b) a BglIII/blunted fragment from F62b (see description of Figure 5A, below) which contains the polylinker with the XhoI, EcoRI, XbaI and ClaI sites.

35 These three clones then provide the possibility for alternative digestions to separate the signal strand from the probe strand.

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C) Production of single-stranded DNA

The plasmid pMLC12/13Δ M71VRTL (see Figure 4D) was transformed into the strain XS127 (argE(am), thi-1, ΔlacproXIII/F'lacproAB, traD36, lacIq, lacZΔ M1S/p3) or
5 JM101 (ATCC #33876) which is used to produce single-stranded DNA from M13 ori plasmids. An overnight culture, (about 3×10^9 cells) was mixed with 5×10^{10} wild type M13 phage, diluted to 100 ml in SOB media and
10 grown for 5 hours at 37°C. Single-stranded DNA was prepared from the culture supernatant using standard procedures. Figure 4E shows diagrammatically the regions of single- and double-stranded DNA which should form in this nucleic acid molecule from its
15 complementary strand.

Digestion of this DNA with BamHI, PstI, XbaI, and EcoRI, alone or in various combinations, produced the predicted fragments.

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Example 2 - Preparation Of Reagent Complex From Replicable Nucleic Acid Molecule

Single-stranded DNA from pMLC12/13A M71VRTL (see Figure 4E) was digested with BamHI and the resulting 3' recessed BamHI end was filled in with alpha α ^{32}P -dATP and cold dCTP, dGTP, TTP. The reaction was incubated at 68°C for 10 minutes to heat inactivate the klenow fragment of DNA Polymerase I and digested to completion with EcoRI. The resulting DNA was chromatographed twice over an hydroxylapatite (HAP) column in 0.12 M phosphate buffer (pH 6.8) to isolate the partially double-stranded labeled molecule from the major portion of the vector. Following binding to the HAP column in 0.12 M phosphate buffer and washing, partially double-stranded labeled construct was eluted from the HAP column with 0.3 M phosphate buffer and concentrated.

Example 3

The isolated reagent complex (about 12 ng) of Example 2 was then incubated in a 10 ul volume of 20mM Tris pH 8, 1M NaCl, 10 mM EDTA, at 65° C for 60 minutes alone or with about 300 ng of single-stranded DNA from M13 clones G7c and G7e, which contain the albumin Hinc III/Pvu II 915 base pair fragment cloned into the Sma I site of the vector Mp8 in opposite orientations or no DNA. The labeled strand was completely displaced from the reagent complex in the presence of the competing strand (G7c) and was not displaced in the absence of competing strand (G7e; no DNA), as measured by gel electrophoresis and autoradiography.

Example 4

Construction of Nucleic Acid Precursor

The double-stranded IV polylinker

BglII EcoRI KpnI
 AGATCTCGAGAATTCTAGATCGATGGTACC
 TCTAGAGCTCTTAAGATCTAGCTACCATGG

was prepared by chemical synthesis and inserted into a

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modified pUC plasmid in both orientations (a and b). The plasmid pUC is described by Viera and Messing, Gene, vol. 19, pp. 259-268 (1982). The pUC plasmid was modified in several ways, the only one of which is relevant to present Example being the addition of an SfiI linker at a site in the plasmid other than the site at which the IV polylinker was subsequently added. The orientation (A) where the SfiI site was closest to the BglII site was digested with SfiI and Kpn I and the small fragment was isolated. The orientation (B) where the Sfi I site was closest to the Kpn I site was digested with Sfi I and Kpn I, and the large fragment was isolated. The gel isolated fragments were ligated and transformed into MC1061. The desired clone, containing two copies of the polylinker in opposite orientation with the KpnI site in the middle was identified by BglII digestion, end labelling with α 32 P-dATP and the klenow fragment of DNA Polymerase I and gel electrophoresis before and after KpnI digestion. The resulting plasmid is termed F58a.

F58a was digested to completion with BglII. The plasmid pMLC12 was digested with Bam HI and treated with Calf Intestinal Alkaline Phosphatase to remove 5' phosphate groups. The two DNAs were ligated and transformed into MC1061 and chloramphenicol resistant colonies were selected. Plasmids which had correctly inserted the BglII fragment with the IV polylinker were identified by ability of ClaI, XhoI, and KpnI to linearize the plasmid. The correct clone is termed F62b (see Figure 5A).

F62b was digested with SacI and HindIII and blunted using the klenow fragment of DNA Polymerase I. The small 89 bp fragment was isolated following gel electrophoresis. The vector pMLC12/13 Δ M7 (see Example 1, part A and Figure 4A) and the 89 bp fragment were ligated and transformed into MC1061. Chloramphenicol resistant colonies were grown up and DNA was prepared. Colonies containing the 89 bp fragment were identified

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by XhoI or KpnI digestion (which will linearize the plasmid) and the orientation of the insert was determined by PstI plus SacI digestion. The two orientations of the plasmid designated pUDV-A and pUDV-B, shown in Figure 5B, were assigned as follows:

5 Orientation A produced two 253 bp SacI/PstI fragments and thus the PstI site was nearest to the 5' end of the CAT gene. Orientation B produced SacI + PstI fragments of 183 and 323 bp and thus the PstI site was farthest from the 5' end of the CAT gene. The nucleotide sequences of the inserts in both orientations of pUDV are given in Figures 1B and 2B, for orientations B and A, respectively.

Example 5

15 If 5' end ligation is for solid support attachment, the target sequence is cloned into the blunted PstI site of pUDV-A, prepared in Example 4. The fragment cloned should have the target binding region TBR including the complement to the signal strand (LBR). The orientation of the first cloning step can be easily determined using

20 either PuvII or SacII (which cut in the vector) and an enzyme which digests the insert DNA. Mini prep DNA is then prepared and digested with SmaI. This provides a blunt end site in which to clone the signal strand fragment. The orientation of the signal strand fragment

25 is determined similarly and an orientation is chosen which is opposite to that of the target strand.

If the 5' end ligation is for signal attachment, then the cloning into the same sites is used for the same fragments but the vector pUDV-B is used.

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Example 6

A similar vector to that prepared in Example 5 could be prepared in which the IV polylinker is replaced by the TL polylinker, whose structure is that described above in connection with plasmid MpTL poly (having EcoRI

35 sites near each end and a StuI site at the middle of the polylinker). This polylinker would serve the purpose of segment 16/16' in Figures 1A and 1B.

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Example 7

The plasmid pUDV-A (see Example 4 and Figure 2B) was digested with the enzyme Pvu II and the 399 nt fragment containing the cloned polylinker sequence was gel isolated (Fragment A). Pvu II cuts at two sites outside of the polylinker, but within the lac region sites shown in Figure 2B. The vector pUC 118, obtained from Dr. Jeffrey Viera, is similar to vector pUC 18 (which is available commercially from New England Biolabs), but contains the M13 origin sequence (nucleotides 5465 (HgiA1 site) - 5941 (Aha I site) of the standard M13 map) inserted at the Nde I site of pUC 18. That vector pUC 118 was digested with PVU II and the 2840 nt fragment containing the plasmid origin of replication, the ampicillin resistance gene and the M13 origin of replication was isolated (fragment B). Fragments A and B were ligated together and transformed into competent bacteria. Colonies containing plasmids with single inserts in the orientation wherein superinfection produces a template that contains (in the clockwise 5' to 3' orientation) amp (including a Bgl I site), Col El ori, Pvu II, mp7, Sma I, hairpin, Xba I, Hinc II, Pst I, mp7, Pvu II, Bgl I, M13 IG were identified by restriction mapping and hybridization with a strand specific oligonucleotide (5'-CGTTGTAAAACGACGGCC-3'). The resulting plasmid was termed p61A and produces a template containing the sequence such as is shown in Figure 1B.

Subcloning of lambda fragments

A map of the relevant region of the bacteriophage lambda genome is given by Sanger, et al in Sanger, et al., J. Mol. Biol 162:301-302 (1982). Of interest are restriction sites of the following types at the following positions.

<u>Site</u>	<u>Position</u>
Pst I	16236
Hae III	16322

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Xmn I	16914
Kpn I	17058
Hinc II	17077
Hae III	17294
Pst I	17395

5 The Pst I fragment of lambda containing this region was isolated by digestion with Pst I and gel electrophoresis. The 86 nt Pst I - Hae III fragment from position 16236 to position 16322 (the A fragment) was subcloned by digesting the isolated Pst I fragment
10 of lambda with Hae III, isolating the 86 nt fragment and blunting the Pst I end with the Klenow fragment of DNA polymerase I. This fragment was then cloned into the Hinc II site of bacteriophage mp7 and the correct clone (mp7lambdaA) identified by hybridization with a specific
15 oligonucleotide and by DNA sequence analysis. Similarly, the 163 nt Xmn I - Hinc II fragment from position 16914 to position 17077 (the D fragment) was subcloned by digesting the lambda Pst I fragment with Xmn I and Hinc II and gel isolating the resulting 163 nt
20 fragment. This fragment was then cloned into the Hinc II site of mp7 resulting in mp7lambdaD.

Cloning into p61A

25 The clone mp7lambdaA (or mp7lambdaD) was digested with Bam HI and the insert fragment was blunted with the Klenow fragment of DNA polymerase I. This fragment was then cloned into the Sma I site of p61A and the desired orientation was selected by hybridization with the strand specific oligonucleotide #1091 (for A; 5'-
30 CCGTTATCCACGATGGCCTC-3') or #1090 (for D; 5'-CATCGCCCGGTACATGGCG-3') resulting in the clones pA6 and pD10, respectively. The entire Lambda Pst I fragment was then inserted into the Pst I site of pA6 or pD10 and oriented by digestion with Kpn I. A map of the clones
35 containing the lambda Pst I fragment in the desired orientations together with a diagram of the expected template folding pattern in the single stranded form of

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these plasmids would show structures analogous to structures represented as Figures 3E (pA61ambda2c) and 3C (pD101ambda1a) except that the 5' and 3' ends become the 3' and 5' ends, respectively.

5 Proposed Use

Clones pA61ambda2c and pD101ambda1a (which contains the 86 nt and 163 nt pairing segments, respectively, but also containing the 1159 nt target binding region) are grown up in E. coli, superinfected with M13 and the
10 harvested DNA is digested with Bam HI (producing constructs analogous to Figures 3E and 3C, respectively). The 5' ends of such constructs are labeled by the procedures of Examples 11 and 12, below, with an oligonucleotide containing a biotin moiety (see
15 U.S.S.N. 729,700 of Brown, et al.). The 3' ends of such constructs are elongated by action of the enzyme terminal deoxynucleotidyl transferase (TdT) with the nucleoside triphosphate dCTP to form an immobilizable oligo-dC tail. The complex can now be cleaved with the
20 restriction endonuclease Eco RI to cleave the hairpin sequence. For assays involving capture by an immobilizable or immobilized strand having a sequence complementary to all or a portion of lambda sequence A (16236-16322) or of lambda sequence D (16914 to 17077),
25 as in U.S.S.N. 809,971 of Dougherty, et al, filed December 16, 1985, such modification of the 3' end and such cutting of the hairpin are optional. Furthermore, if a biotinylation or other labeling technique not requiring free ends is employed, the harvested DNA prior
30 to BamHI cleavage can be used for assays employing displacement and capture of the displaced pairing segment.

Example 8

35 The cloning vector p61a described in Example 7 was used in this Example. Sequences from the cDNA sequence for erythropoietin (EPO) were used in this Example for

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target binding region, pairing segment and analyte. The relevant cDNA region, clone pG61aEPO3III, has the map shown by Jacobs, et al in Nature, vol. 313, pp. 806-810 (1985) including:

	<u>Site</u>	<u>Position</u>
5	Eco RI	1 (inserted 6 bases upstream from initiator methionine codon)
	Kpn I	133
	Pst I	188; 363
	Hinc II	335
10	Pvu II	365

The 175 base pair Pst I fragment (188 - 363) was cloned into the Pst I site of p61a plasmid DNA. Clones containing the insert were identified by hybridization to a kinased oligonucleotide probe. Single stranded
15 plasmid DNA was prepared from positive colonies after superinfection with an M13 helper phage. The orientation of the Pst I fragment in the vector was determined by binding this single stranded DNA to
20 nitrocellulose filters and hybridizing it with kinased oligonucleotide probes of opposite orientations. Plasmid p61A-EPO3 contains the Pst I fragment in the orientation with the Hinc II site closest to the 3' end of the insertion.

Double stranded plasmid DNA was isolated from p61A-EPO3 and digested with Sma I. The 365 base pair Eco RI-Pvu II fragment (1 - 365) from the human EPO cDNA clone was gel purified and the Eco RI site was filled in with the Klenow fragment of DNA Polymerase I. This fragment
25 was then ligated into the Sma I site of p61A-EPO3; plasmids containing inserts were identified by screening
30 colonies with a kinased oligonucleotide probe specific for this second insert. The orientation of the second insert was determined by analyzing Kpn I digests of the resulting double stranded plasmid DNA. Those plasmids
35 with the target binding region and pairing segments in an inverted orientation contain a Kpn fragment of 166 base pairs, while those in the direct orientation

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contain a Kpn fragment of 264 base pairs. p61A-EPO3-II was identified as having the target strand inverted with respect to the signal strand and was isolated for preparation of displacement complexes.

5 Single stranded p61A-EPO3-II DNA was prepared as described in Example 1, except that the overnight culture was superinfected with the M13 phage derivative, MK107 (see Example 9) diluted to 100 ml in minimal media containing 50 ug/ml ampicillin and grown overnight at 37°C. The DNA was digested with Bam HI and the 3' end 10 (near the 175 nt pairing segment) labeled with 32P-dATP using the Klenow fragment of DNA Polymerase I. The DNA was then digested with Eco RI to create final displacement complexes, by analogy with Figure 1F.

15 The 370 base pair Eco RI-Pvu II fragment described above was also cloned into the Acc I site of the M13 phage vector mp7 after both the Acc I site and the Eco RI site were filled in with Klenow. Phage containing EPO inserts, and the orientation of those inserts, were identified by hybridization with kinased oligonucleotide 20 probes. Single stranded template DNA was prepared from positive phage of each orientation and digested with Bam HI. The 370 nucleotide inserts were gel purified and used as model analytes.

25 Three displacement reactions were set up using this complex and either no analyte, excess analyte of the wrong orientation, or excess analyte of the correct orientation. No displacement was observed in the absence of analyte or with analyte of the wrong orientation. Displacement was observed with analyte of 30 the proper orientation, although the reaction did not go to completion due to incomplete digestion of the complex with Eco RI; such complexes bind analyte but do not release the signal strand from the probe strand after displacement.

35

Example 9-13

This example and the examples that follow are illustrations of the capture methodology of U.S.S.N.

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809,971 of Dougherty, et al, filed December 16, 1985, and correspond to certain of Examples 2-10 of that application. The starting clone was pMLC12/13deltaM7IVRTL, whose preparation is described above in Example 1, and whose structure is shown in
5 Figure 4D (double-stranded form) and Figure 4E (single-stranded form).

The construct p66b was constructed by gel-isolating the double-stranded PvuII fragment containing the
10 sequence of interest from the starting clone and ligating it to the gel-isolated Pvu II backbone of the M13 origin plasmid pUC 119 (similar to commercially available plasmid PUC 19, but containing the same M13 origin sequence as does pUC118, and also obtained from
15 Dr. Jeffrey Viera). Single-stranded forms were obtained as described in Example 1, except that the DNA was transformed into the E. coli host strain MV1193 obtained from Dr. Michael Volkert (JM101 del(srlR-recA) 306::Tn10). Superinfection was with bacteriophage
20 M13K07. The use of the different pUC backbone, different E. coli host strain and different superinfecting M13 phage resulted in improved yields of the same construct after Bam HI digestion.

Inverse reagent complexes used in Examples 12 and 13 resulted from labelling the 5' end of p66b with P32.
25 p66d was constructed as described for p66b, except that the PvuII fragment was inserted into the pUC119 vector in the opposite orientation. The single stranded form of p66d produced after superinfection contains a displacement complex which is the complement of the p66b
30 strand. Thus, before cleavage in the IVRTL hairpin, displacement complexes made from p66b contain the signal strand at the 5' end and the target binding region at the 3' end.

II. Model Analytes: Model analytes were
35 constructed by gel purifying a 2 kb Hind III-Eco RI fragment from a plasmid, pAllAlb, which contains the entire cDNA sequence of human albumin. The HindIII site

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is the HindIII site in the 3' end of the albumin cDNA. The EcoRI site is present in adjacent vector sequences. The vector sequences present on the HindIII EcoRI fragment have no bearing on the following examples. The HindIII - EcoRI fragment was ligated into
5 Hind III-EcoRI digested M13mp8 and M13mpl1 to give mp8AllAlb and mpl1.AllAlb, respectively. Single stranded DNA was purified from phage containing these constructs, and was partially digested with Hae III to
10 linearize these model analytes. There are no HaeIII sites within the albumin cDNA sequence. mp8.AllAlb template DNA is complementary to the target binding region of p66b displacement complexes, and mpl1.AllAlb (see Figure 4 of U.S.S.N. 809,971), template DNA is
15 complementary to the TBR of p66d displacement complexes.

III. Model Capturers: Several different model capturers were constructed to help define the best geometry for capturing nucleic acid constructs.

c1. mp18.AlbTaqPst and mpl9.AlbTaqPst were constructed by gel purifying a 350 base pair Bgl II-Pst
20 I fragment from a human albumin cDNA clone, digesting it with Taq I, and ligating the resulting 280 base pair fragment into Acc I-Pst I mp18 and mpl9 vectors. mp19.AlbTaqPst is complementary to the pairing segment
(PS') of the labeled strand of p66b. mp18.AlbTaqPst is
25 complementary to the pairing segment in p66d.

c2. mp8.AlbBH was made by ligating the 500 base pair Bgl II-Hinc II fragment from albumin cDNA into the
Sma I site of mp8. The insert in mp8.AlbBH is
30 coextensive with and complementary to the pairing segment in the p66b displacement complex described above.

c3. mp7delta.AlbXba constructs 1+, 2+, 3-, and 4- were made by digesting mpl9.AlbTaqPst Rf DNA with Xba I and end filling, gel purifying the resulting 300 base
35 pair fragment, and ligating it to the 6800 base pair gel purified Pvu II vector backbone fragment of mp7. Two resulting phage isolates containing single stranded

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albumin DNA complementary to the labeled polynucleotides of p66b displacement complexes are labeled 1+ and 2+, while two phage isolates containing the albumin strand complementary to probe polynucleotide strands of p66b are labeled 3- and 4-. Constructs 1+ and 2+ differ from mp19.AlbTaqPst in that a portion of the lac gene and all polylinker cloning sequences are deleted from the mp7delta backbone, and in that the albumin insert is complementary to a more interior portion of the signal strand (see Figure 4 of U.S.S.N. 809,971).

c4. Biotinylation of mp7deltaAlbXbal+ and mp7deltaAlbXba3- DNA using Vector Laboratories Photoprobe™ Biotin.

The capturing strands mp7deltaAlbXbal+ or mp7deltaAlbXba3- were biotinylated using the commercially obtained Photoprobe Biotin (Vector Laboratories) essentially as described by the manufacturer and repeated below.

Photoprobe™ biotin (500 ug) was resuspended in 500 microliter water as recommended by the manufacturer and stored in the dark at -20°C. 10 micrograms of template DNA from the clone mp7 deltaAlbTaqXbal+ or mp7deltaAlbXba3- were ethanol precipitated and resuspended in 10 microliter H₂O. The DNA was mixed with 10 microliter Photoprobe biotin solution under a safelight, sealed in a glass microcapillary pipette and irradiated by a sunlamp (GE infrared lamp 2JOR40/1) for 20 or 30 minutes in separate reactions. The sample was kept in an ice-H₂O bath during the entire irradiation procedure. After irradiation, the sample was removed from the capillary, diluted with 100 microliters of 0.1 M Tris-HCl, pH 8.0, extracted twice with 2-butanol and precipitated following addition of 1/10 volume 3M Na Acetate and 2 volumes of ethanol. The precipitated sample was resuspended in 10 microliters 0.1 mM EDTA, pH 8.0.

Successful reaction was monitored by taking an aliquot of the biotinylated DNA and hybridizing a 32-P

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labeled oligonucleotide (cALB 32-mer) complementary to a 32 base segment of the capture strand. One-half of the sample (control) was then electrophoresed directly on an agarose gel. The other half was mixed with 10 microliter of streptavidin latex beads supplied from Pandex Laboratories in 0.2 M NaCl, 20 mM Tris-HCl, pH 8.0, 0.1% NP-40 for 10-20 minutes at room temperature. After the binding step, the beads were removed from the solution by centrifugation (2 minutes, Eppendorf centrifuge) washed once and the combined solution phases were electrophoresed in a parallel lane to the control sample. Following electrophoresis and autoradiography, the results indicated that nearly all the 32-P labeled oligonucleotide sample that was hybridized to the mp7deltaAlbXbaI+ DNA was removed from the sample that was exposed to the streptavidin latex, indicating that the majority of template DNAs (capture strands) had at least one biotin group attached.

Example 10: Labeling of p66b Xba I complex by ligation of a kinased oligonucleotide results in decreased non-specific capturing.

In this experiment, p66b Xba complex was labeled by ligating a kinased oligonucleotide to the 3' end of the molecule. 10 pm of a 15 base oligonucleotide with the sequence 5' CTAGAGGCCTCTGCA3' was labeled at the 5' end with 32P -gamma ATP and polynucleotide kinase (see Maniatis et al, Cloning Manual (Cold Spring Harbor Laboratory 1982)). The labeled oligonucleotide was purified away from unincorporated 32P - gamma ATP by centrifuging the reaction twice at 6000 rpm for 30 minutes in a total volume of 500 ul of TE in a Centricon 10 filtration device from Amicon. The kinased oligonucleotide and 1 pm of Xba I cut p66b gel purified complex were precipitated together with ethanol, and resuspended in 14 ul of TE. 4 ul of 5X ligase buffer (Maniatis et al Manual) and 2 ul of DNA ligase were added and the reaction was incubated at 15° for 4 hours. The reaction was diluted to 50 ul with TE,

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heated to 42°C for 10 minutes to melt any non-ligated oligonucleotides and electrophoresed on a 1% agarose gel. Following electrophoresis, the gel was stained with ethidium bromide and viewed with a uv light box. Approximately 66% of the complexes ligated to each other, so that only 0.33 μ m of complex were available for ligation to the kinased oligonucleotide. The kinased complex was purified and had an estimated specific activity of about 10^6 cpm per μ m.

Displacement and capturing reactions were carried out by incubating with either 0.001 μ m (1000 cpm) or 0.005 μ m (5000 cpm) of displacement complex with varying amounts of analyte and 0.2 μ m of capturer in a total of 20 μ l of 0.3 M NaCl and 0.1 M Tris HCl, pH 8.0, for 45 minutes at 65°C. In the absence of analyte, no non-specific capturing was observed in reactions with 0.001 μ m of complex and 0.02 μ m of either mp7, mp19, mp7deltaAlbXba3- or mp19.AlbTaqPst. The addition of 0.01 μ m of analyte to these reactions resulted in 100% displacement, and in no detectable capturing in reactions with mp7, mp19, or mp7deltaAlbXba3-, and in greater than 90% captured complexes with mp19.AlbTaqPst. In reactions with 0.005 μ m of complex, in the absence of analyte no detectable capturing was observed with mp7deltaAlbXba3-, less than about 0.5% non-specific capturing was observed with mp7, mp19 and mp19.AlbTaqPst. Slightly more background was observed with mp19.AlbTaqPst than with mp7 and mp19. In the presence of 0.01 μ m of analyte, less than 1% capturing was observed with mp7, mp19, or mp7delta.AlbXba3-, while greater than 90% capturing was observed with mp19.AlbTaqPst.

These results indicate that lower background levels for non-specific capturing can be obtained using complexes which are labeled by ligation to a kinased oligonucleotide (or other techniques not causative of nicks). In addition, the absence of nonspecific capturing with mp7deltaAlbXba3- indicates that non-

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specific capturing at experimentally detectable levels is not an inherent property of the capturing concept.

Example 11: Displacement and capturing with Bam HI p66b and mp7deltaAlbXba capturers.

5 Single stranded p66b DNA was digested to completion with Bam HI, and the covalent complex was isolated by gel purification. A 32P-kinased oligonucleotide with the sequence 5'GATCCGCGGCGGTAC3' was ligated to the 3' end of the complex as described in Example [10] except that 2.4 μ m of the complex and 10 μ m of the
10 oligonucleotide were used in the ligation reaction. The specific activity of the resulting complex was estimated at 8×10^4 cpm/ μ m.

Reactions were done by incubating either 0.01 μ m (1000 cpm) or 0.03 μ m (3000 cpm) in the presence or
15 absence of 0.01 μ m Hae III cut mp8.AllAlb analyte, with 0.1 μ m of capture DNA in a final volume of 20 μ l of hybridization buffer (0.3M NaCl, 0.1 M Tris HCl, pH8.0, and 10 mM EDTA) for 60 minutes at 65°C. Reactions were
20 analyzed by gel electrophoresis (1% agarose gel) and autoradiography.

In the absence of analyte, no detectable background capturing was observed with any of the following capturers: mp7, mp19, mp7deltaAlbXbal+, mp7deltaAlbXba3- or mp7deltaAlbXba4-. The addition of
25 0.01 μ m of analyte to reactions with 0.01 μ m complex resulted in 100% displacement; no capturing was observed with the mp7deltaAlbXba4- capturer, and 100% capturing was observed with the mp7deltaAlbXbal+ capturer. The
30 addition of 0.01 μ m of analyte to reactions with 0.03 μ m complex resulted in approximately 50% displacement and no capturing with mp7deltaAlbXba4- and complete capturing of the displaced complexes with mp7deltaAlbXbal+.

Example 12: Comparision of displacement and capturing with covalent and non-covalent p66d complexes.

35 Covalent p66d displacement complexes were prepared and labeled at the 5' end by ligation of the kinased

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16mer using the EF21 splint as described in Example 13. The specific activity of the resulting complexes was about 1×10^6 cpm/pm.

Non-covalent p66d complexes were produced by complete digestion of approximately 50 ug of single stranded templated DNA with Bam HI and Eco RI. Complete digestion was ascertained by the appearance of equimolar amounts of three bands, corresponding to vector backbone, target strand, and signal strand, after electrophoresis of a small aliquot of the digest on an alkaline gel. Non-covalent p66d complexes were labeled at the 5' end of the signal strand as described for covalent complexes, by EF21 splint ligation of a kinased 16mer, with a resultant specific activity of 3×10^6 cpm/pm.

The four reactions outlined in Table 3 were set up in a total volume of 50 ul of hybridization buffer and incubated for 60 minutes at 65°C using HaeIII digested mp11.AllAlb DNA as analyte and biotinylated mp7deltaAlbXba3- capturer. 10 ul of each reaction were analyzed by gel electrophoresis and autoradiography, and the remaining 40 ul by binding to streptavidin agarose. 200 ul packed volume streptavidin agarose was used per reaction. Binding and washing was as described in Example 13 (below), except that, after binding, the pellet was rinsed 3 times for 30 minutes at room temperature and once for 60 minutes at 65°C. The final pellet and all supernatants were counted. Data showing the cpm bound to agarose after each rinse are given in Table 1. These results show that displacement and capturing are approximately equally effective for covalent and non-covalent complexes.

Gel analysis of the same reactions, as well as two reactions in which only displacement complex and capturer were included, demonstrated that 100% of the complexes were displaced by analyte, and when capturer was included, 100% capturing occurred. In the absence of analyte, no capturing was observed. In addition,

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since complexes which have hybridized both to capturer and analyte (i.e., captured covalent complexes or the second intermediate) migrate differently from capturers which have hybridized only to signal strand displaced from noncovalent complexes, one can distinguish capturing intermediates ("second intermediates") which contain capturer hybridized to analyte which in turn is hybridized to the target binding region of the displacement complex, from those non-covalent complexes which have resolved to contain only capturer and displaced signal strand. In this experiment, approximately 90% of the captured signal is present in the resolved form, despite the fact that capture DNA was present in excess over analyte and complex, and would be likely to form the intermediate structure before displacement.

TABLE 1

	<u>REACTION</u>	<u>pm COMPLEX</u>	<u>pm ANALYTE</u>	<u>pm CAPTURER</u>
20	1	0.2 covalent	0.2	0.8
	2	0.2 noncovalent		0.2
	3	0.2 covalent	0.2	0
25	4	0.2 noncovalent		0.2

CPM BOUND TO SUPPORT

	<u>REACTION:</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
30	TOTAL:	9821	26885	9318	30558
	RINSE 1:	7932	24406	2403	5625
	RINSE 2:	7383	22793	1267	1859
	RINSE 3:	7055	20363	827	956
	RINSE 4:	6186	19063	666	512
35	FINAL	5562	16718	304	299
	%Bound:	55.6%	62.1%	1.7%	1.0%

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Example 13: Large scale displacement and capture with trapping on streptavidin agarose.

The Bam p66b displacement complex was labeled to a specific activity of about 10^6 cpm/ μ m by ligating a 32 P-kinased oligonucleotide to the 5' end of the complex with the use of a 21 base splint (EF21). 10 μ m of the kinased 16mer (indicated below by the asterisk), 10 μ m of splint, and 1 μ m of p66b Bam (underlined below) were incubated together at 22°C for 15 minutes in 10 μ l of 1X ligase buffer; 1 μ l of ligase was added and the reaction incubated for an additional 30 minutes. The three molecules form the structure diagrammed below.

*CGAAGCTTGGATCCGCGATCCGTCAGCTT...p66b

GAACCTAGGCGCTAGGCAGCT (SPLINT)

The four reactions outlined in Table 2 were set up in a total volume of 50 μ l of hybridization buffer (see Example 14) and incubated for 30 minutes at 65°C. Hae III cut mpAllAlb and biotinylated mp7delta.AlbXba DNA were used as analyte and capturer, respectively. 25 μ l of each reaction was then analyzed by gel electrophoresis and 25 μ l by binding to streptavidin agarose as follows. 100 μ l packed volume of streptavidin agarose was washed twice in 500 μ l binding buffer in a 5 ml Sarstedt tube rotated end over end for 15 minutes, and pelleted by centrifugation. The 25 μ l reaction aliquots were diluted to a total of 500 μ l binding buffer, and incubated, rotating as above, for 15 minutes. The sample was transferred to an Eppendorf tube for centrifugation, the supernatant saved, and the pellet rewashed as above, once at room temperature for 15 minutes, then twice at 65°C for 15 minutes, then for 60 minutes at room temperature and finally for 15 minutes at room temperature with TE. The final pellet and all supernatants were counted. Data showing the cpm bound to agarose after each rinse are given in Table 2. These results show that binding of complex to the support is dependent upon the presence of capturer and

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analyte, and on the amount of analyte present.

Gel analysis of the same reactions indicated that there is less than 0.05% non-specific capturing in these reactions. Specific capturing was more efficiently analyzed by gel separation, in that the presence of
 5 analyte resulted in capturing of approximately 80% and 20% of the complex in reactions 3 and 4, respectively.

It should be noted that this Example 13 and Example 14, below, provide labeling at the 5'-end, and thus adjacent to the target binding region rather than to the
 10 pairing segment. Such a geometry is described further in a copending application of Collins et al, U.S.S.N. 809,992, filed December 16, 1985.

TABLE 2				
15	<u>REACTION</u>	<u>pm COMPLEX</u>	<u>pm ANALYTE</u>	<u>pm CAPTURER</u>
	1	0.10	0	0
	2	0.10	0	0.16
	3	0.10	0.05	0.16
20	4	0.10	0.01	0.16

CPM BOUND TO SUPPORT

25	<u>REACTION:</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
	TOTAL	46435	42590	49423	41889
	RINSE 1:	12915	10150	274308	15394
	RINSE 2:	5254	3217	22982	10109
	RINSE 3:	3911	2032	19978	7806
30	RINSE 4:	3500	1594	16261	6238
	RINSE 5:	2203	1458	13780	5391
	RINSE 6:	1482	1227	12351	4436
	FINAL:	813	1044	10628	3658
	% BOUND:	1.7	2.5	26.3	8.7

35 Example 14: Prehybridization of complex and analyte, followed by capturing and trapping.

Two additional reactions were done using the Bam

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p66b complex described in Example 13. In these reactions, 0.1 μ m complex alone (reaction 1) or 0.1 μ m complex and 0.05 μ m Hae III cut mp8AllAlb analyte (reaction 2) were incubated in 50 μ l of hybridization buffer for 30 minutes at 65°C. 0.16 μ m of biotinylated mp7deltaAlbXbal+ was then added to both reactions, which were then divided and treated as in Example 13, except that all rinses were at room temperature with binding buffer. By gel analysis, less than 0.05% non-specific capturing, and approximately 40% specific capturing was observed. The results of analysis on streptavidin agarose (Table 3) indicate that capturing and trapping occur with approximately equal efficiencies whether capture DNA is added after (as in this Example 14) or is present during (as in Example 13) the analyte-dependent displacement reaction.

TABLE 3
CPM BOUND TO SUPPORT

REACTION	1	2
TOTAL:	57860	55210
RINSE 1:	10364	18538
RINSE 2:	4360	14321
RINSE 3:	3046	11983
RINSE 4:	3035	9745
RINSE 5:	1781	8897
FINAL:	1124	7428
% BOUND:	1.9	13.5

Example 15: Strand displacement, capturing and biotin displacement.

In this experiment, the non-covalent p66d Bam HI/EcoRI cut displacement complex described in Example 12 was used. Hae III cut mp11.AllAlb DNA was used as the analyte. A capturer which has a single biotin at

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the 5' end was synthesized by primer extension of a 5' biotinylated M13 sequencing primer (biotinylated according to the method described in U.S.S.N. 729,700 of Brown, et al.) hybridized to mpl9.AlbTaqPst as follows: 20 ug of mpl9.AlbTaqPst template, and 20 pm of biotinylated primer in 100 ul of 50 mM NaCl, 10 mM Tris HCl, pH8.0, 10 mM MgCl₂ were boiled for 1' in a water bath and allowed to cool to room temperature for 30' in the bath. 3 ul of 5 mM dGTP, dCTP, dATP, dTTP and 2 ul of Klenow fragment Pol I were added, and the reaction incubated 30 minutes at room temperature. A second 1 ul aliquot of Klenow was added and the reaction incubated for 30 additional minutes. The DNA was then digested with Hind III for 2 hours at 37°C to cut out the 300 base primer extended fragment which is complementary to the insert in mpl9.AlbTaqPst. The DNA was denatured by adding 2 ul of 5 M NaOH and incubating it for 10' at 65°C. The primer extended fragment was purified after separation by electrophoresis on a 1% alkaline agarose gel with NA45 paper (Schleicher and Schuell). DNA yield was estimated by comparison of an aliquot of the capturer with standards on an ethidium stained gel.

Three reactions were set up as shown in Table 4. Reactions 1 and 2 were incubated for 60 minutes in 50 ul of hybridization buffer at 65°C (reaction 3 was not incubated). 5 ul of reactions 1 and 2 were then removed for gel analysis. The rest of reactions 1 and 2, and reaction 3 were then bound as described in Example 8 to approximately 200 ul packed volume streptavidin agarose. In order to try and minimize sample agitation during the rinses, which may be responsible for a portion of the captured DNA separating from the support in earlier experiments, the samples were rinsed by adding 1 ml of binding buffer to the pellet in an eppendorf tube, inverting the tube five times, and centrifuging it for 3 minutes. Five rinses were done at room temperature, and a final rinse was done at 65°C for 30 minutes with no shaking after the initial 5

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inversions. All supernatants and the final pellet were counted. The results are shown in Table 4. As seen by these data, this gentler washing protocol, or the use of this new smaller and singly biotinylated capturer seems to promote more stable binding of the captured complex to the support. By cutting out and counting the appropriate bands from the gel analysis, it appears that approximately 36% of the captured complexes resolve to form capture-signal strand hybrids, while 64% are apparently present as analyte-complex-capturer intermediates under these reaction conditions.

TABLE 4

<u>REACTION</u>	<u>pm COMPLEX</u>	<u>pm ANALYTE</u>	<u>pm CAPTURER</u>
1	0.2 noncovalent	0.2	0.5
2	0.2 noncovalent	0	0.5
3	0.2 noncovalent	0	0

CPM BOUND TO SUPPORT

<u>REACTION:</u>	<u>1</u>	<u>2</u>	<u>3</u>
TOTAL:	25623	25524	29269
RINSE 1:	13618	2884	4802
RINSE 2:	12214	723	1927
RINSE 3:	11966	519	1511
RINSE 4:	11822	454	954
RINSE 5:	11693	391	845
FINAL	11174	233	687
% BOUND:	43.6%	0.9%	2.3%

Biotin displacement of captured displaced strands.

Reaction 1 to 3 as described above were used. Each sample was the final avidin-agarose pellet after the 30 minute, 60°C wash described above. One ml BB containing

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0.1% NP-40 (BB+) was added to each pellet at room temperature, shaken briefly by inversion and centrifuged to separate the phases (RT wash). One ml BB+ (at 65°C) was then added and the samples were incubated for 5 minutes at 65°C. The samples were then centrifuged to
 5 separate the phases and washed twice with one ml BB+ at room temperature. The combined supernatant phases were then pooled (65°C/5'/-bio). The -biotin, 65°C wash was repeated once more for sample 3 only. One ml of BB+ containing 1 mM biotin was then added to all samples and
 10 these were incubated for 5 minutes, centrifuged and washed as above (65°C/5'/+bio). The final pellet was resuspended in 3 ml BB+ and all samples were counted by Cherenkov counting. Table 5 presents the number of
 15 counts and the percentage (in parenthesis) of total counts recovered where applicable in each sample after the background (30 cpm) was subtracted.

TABLE 5

	1 %	2 %	3 %
20 TREATMENT			
RT Wash	42	2	57
65°C/5'/-bio	182	16	103
65°C/5'/-bio	-	-	-
65°C/5'/+bio	7088 (84%)	70 (54%)	48 (6%)
25 Pellet	1151	42	516
Total	8463	130	778

The signal to noise ratio before the biotin displacement can be defined as the ratio of counts in the reaction 1 pellet/reaction 2 pellet =48:1
 30 (11174/233). The improvement brought about by the biotin displacement can be measured by the ratio of % counts released by biotin in reaction 1 over the percent counts released by biotin in either reaction 2 or 3. Thus by
 35 comparison with reaction 2 the improvement is $0.84/0.54 = 1.55$ X. For reaction 3 the improvement is $0.84/0.06 = 14$ X. The poor improvement seen in the reaction 2 sample is likely due to the fact that the reaction 2

-55-

capturer contains a small amount of M13 polylinker sequence which does result in some capturing by the biotinylated capturer in the absence of analyte. This capturing, though small, would lead to counts released by biotin. The reaction 3 sample (complex only) represents the type of background most likely to be found in an actual displacement measurement and therefore gives a better representation of the background improvement expected.

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We Claim:

1. A nucleic acid construct useful in preparing reagents for determining target nucleotide sequences in the nucleic acid of a biological sample, the construct comprising:

- 5 (a) a target binding region substantially complementary to the target nucleotide sequence, and
(b) a signal strand pairing segment bound in the construct by complementary base pairing to a portion of the target binding region;

10 a second portion of the target binding region being single-stranded; and

the target binding region and signal strand pairing segment being covalently linked by a phosphate/sugar backbone.

15 2. The nucleic acid construct of claim 1 wherein all of the nucleotides bound by complementary base pairing to the signal strand pairing segment are a subset of the nucleotides of the target binding region.

20 3. The nucleic acid construct of claim 1 wherein the nucleotides bound by complementary base pairing to the signal strand pairing segment are adjacent to one end of the target binding region.

25 4. The nucleic acid construct of claim 1 being a substantially single-stranded loop having a 5' end and a 3' end, the signal strand pairing segment being adjacent to one of the ends and the target binding region being adjacent to the other end of the single-stranded loop.

30 5. The nucleic acid construct of claim 4 wherein the nucleotides bound by complementary base pairing to the signal strand pairing segment are adjacent to the other end of the single-strand loop.

35 6. The nucleic acid construct of claim 5 wherein the one end is the 3' end and the other end is the 5' end.

7. The nucleic acid construct of claim 4 wherein the one end is the 3' end and the other end is the 5' end.

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8. The nucleic acid construct of claim 1 further comprising a DNA hairpin loop restriction site between the target binding region and the signal strand binding region along the phosphate/sugar backbone.

5 9. The nucleic acid construct of claim 1 further comprising a detectable tag adjacent to the signal strand pairing segment and distal from the target binding region along the phosphate/sugar backbone.

10 10. The nucleic acid construct of claim 1 being DNA.

11. The nucleic acid construct of claim 1 wherein the region of complementary base pairing between the signal strand pairing segment and a portion of the target binding region is about 15 to about 1000 base pairs in length.

15 12. The nucleic acid construct of claim 11 wherein the region of complementary base pairing between the signal strand pairing segment and a portion of the target binding region is about 20 to about 500 base pairs in length.

20 13. A process for preparing a reagent complex for determining a target nucleotide sequence in the nucleic acid of a biological sample, which process comprises the steps:

25 (a) producing by replication a continuous nucleic acid molecule predominantly in single-stranded form having:

30 (i) a target binding region substantially complementary to the target nucleotide sequence, and

(ii) a signal strand pairing segment bound by complementary base pairing to a portion of the target binding region;

35 a second portion of the target binding region being single-stranded, and the target binding region and signal strand binding region being covalently linked by a phosphate/sugar backbone;

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(b) forming a first terminus adjacent to the signal strand pairing segment and a second terminus adjacent to the target binding region; and

5 (c) attaching a detectable tag to the nucleic acid strand having the first terminus and second terminus.

14. The process of claim 13 wherein the detectable tag is attached to a terminus adjacent to the signal strand pairing segment.

10 15. The process of claim 14 further comprising the step:

(d) cleaving the phosphate/sugar backbone covalent linkage of the signal strand pairing segment to the target binding region.

15 16. The process of claim 15 further comprising the step:

(e) attaching an end of the nucleic acid molecule adjacent to the target binding region to a solid support.

20 17. The process of claim 15 wherein the attaching step (e) precedes the cleaving step (d) and the end attached is distal from the signal strand pairing segment.

25 18. The process of claim 15 wherein the attaching step (e) follows the cleaving step (d) and the end attached is created by the cleaving step (d).

19. The process of claim 14 further comprising the step:

30 (d) attaching a moiety to the nucleic acid molecule at a terminus adjacent to the target binding region, the moiety being immobilizable by an affinity reagent.

35 20. The process of claim 14 wherein the first terminus and second terminus created in forming step (b) are a 3' end and a 5' end, and the attaching step (c) comprises selectively attaching the tag to the first terminus.

21. The process of claim 21 wherein the first

-59-

terminus is a 3' end and the attaching step (c) comprising elongating the 3' end with detectable nucleotides.

22. The process of claim 20 wherein the second terminus is a 5' end and the process further comprises
5 attaching the 5' end to a solid support.

23. The process of claim 20 wherein the second terminus is a 5' end and the process further comprises attaching the 5' end to a moiety which is immobilizable by an affinity reagent.

10 24. The process of claim 13 wherein the detectable tag is attached to a terminus adjacent to the target binding region.

15 25. A replicable nucleic acid useful in preparing reagents for determining target nucleotide sequences in the nucleic acid of a biological sample, having one continuous strand comprising:

- (a) an origin of replication,
- 20 (b) a target binding region substantially complementary to the target nucleotide sequence;
- (c) a signal strand pairing segment substantially complementary to a portion of
25 the target binding region and oriented in an inverse direction compared to the target binding region; and
- (d) two half-restriction sites capable of forming a restriction site and defining
30 therebetween a length of nucleic acid containing the target binding region and the signal strand pairing segment.

26. The replicable nucleic acid molecule of claim
35 25 being in double-stranded form with its complementary strand.

27. The replicable nucleic acid molecule of claim
25 being in predominantly single-stranded form free of

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its complementary strand.

28. The replicable nucleic acid of claim 27 wherein the two half-restriction sites are hybridized to form a restriction site, the length of nucleic acid forms a loop connected at each end to one of the half-
5 restriction sites and the signal strand pairing segment is bound by complementary base pairing to a portion of the target binding region within the loop.

29. The replicable nucleic acid molecule of claim
10 28 wherein signal strand pairing segment and the portion of the target binding region are each adjacent to one of the half restriction sites.

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FIG. 1A 1/8

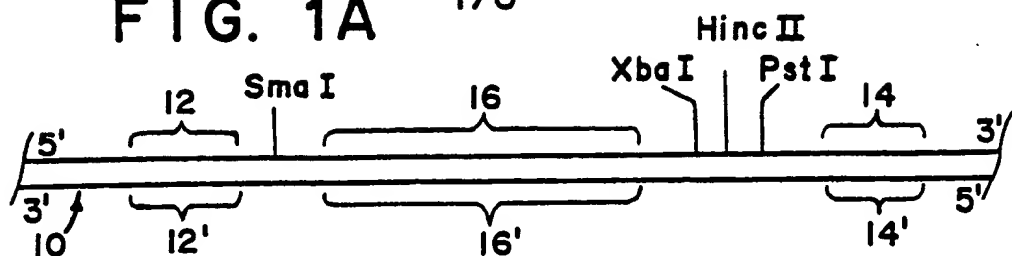


FIG. 1C

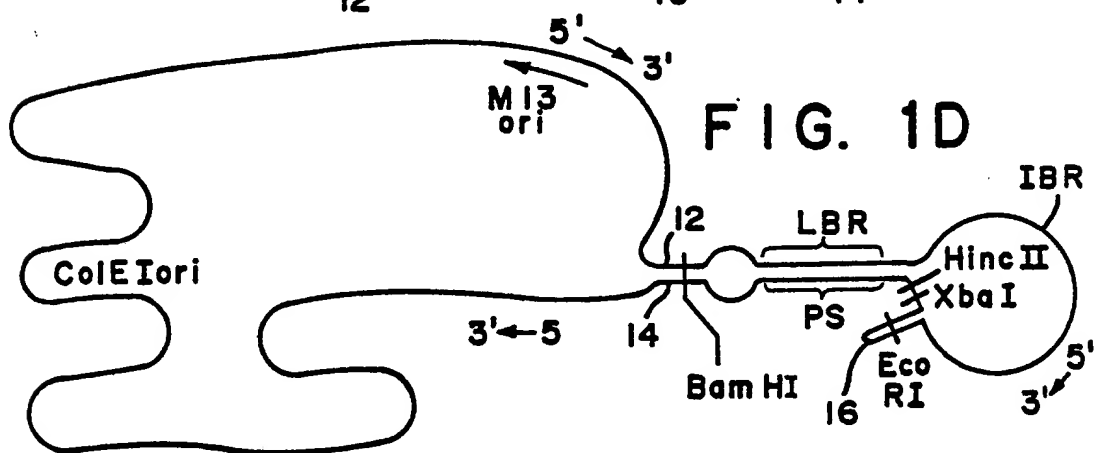
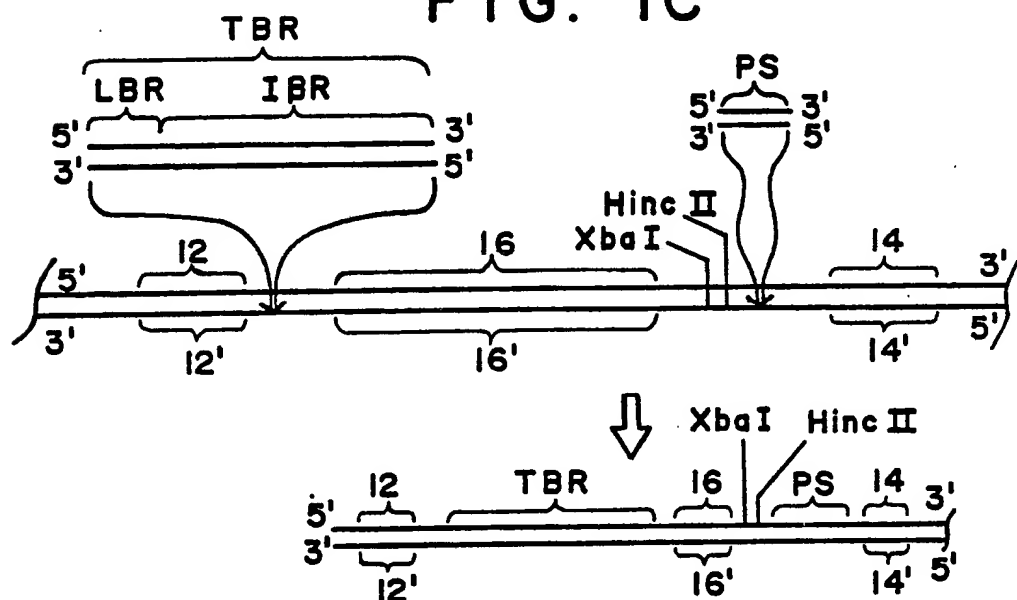


FIG. 1E

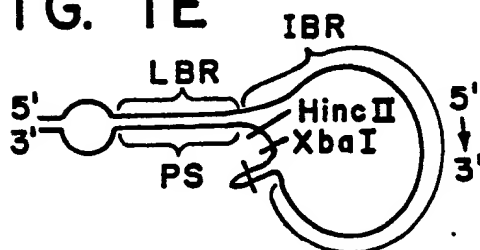
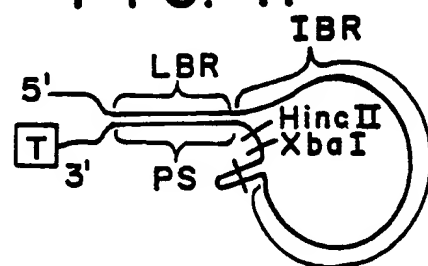
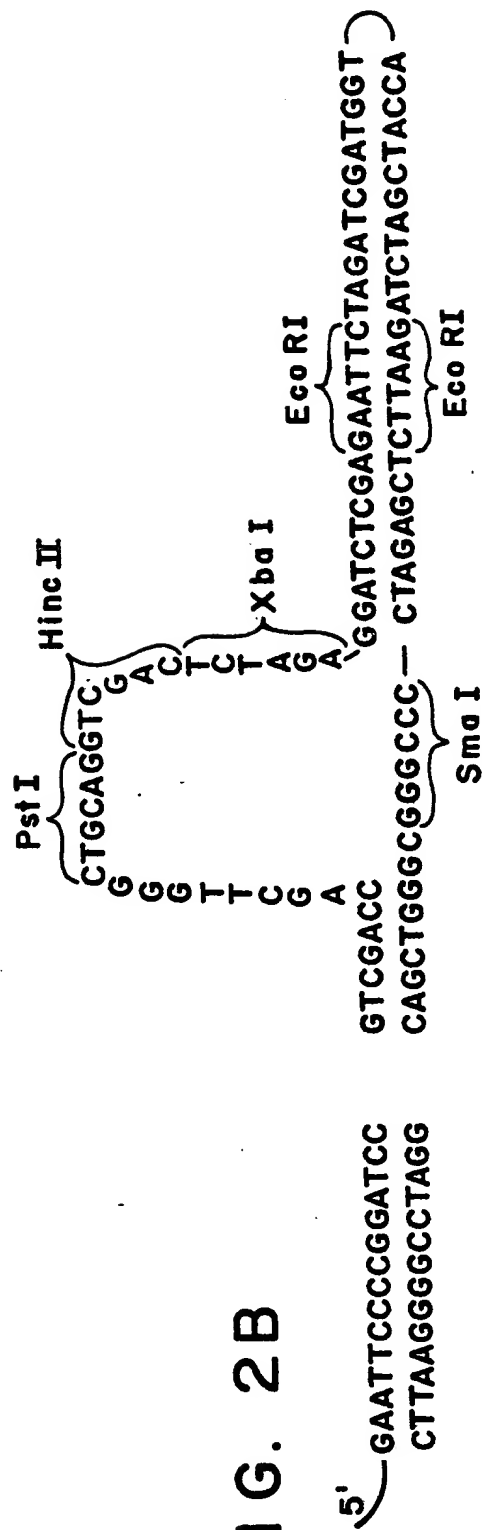
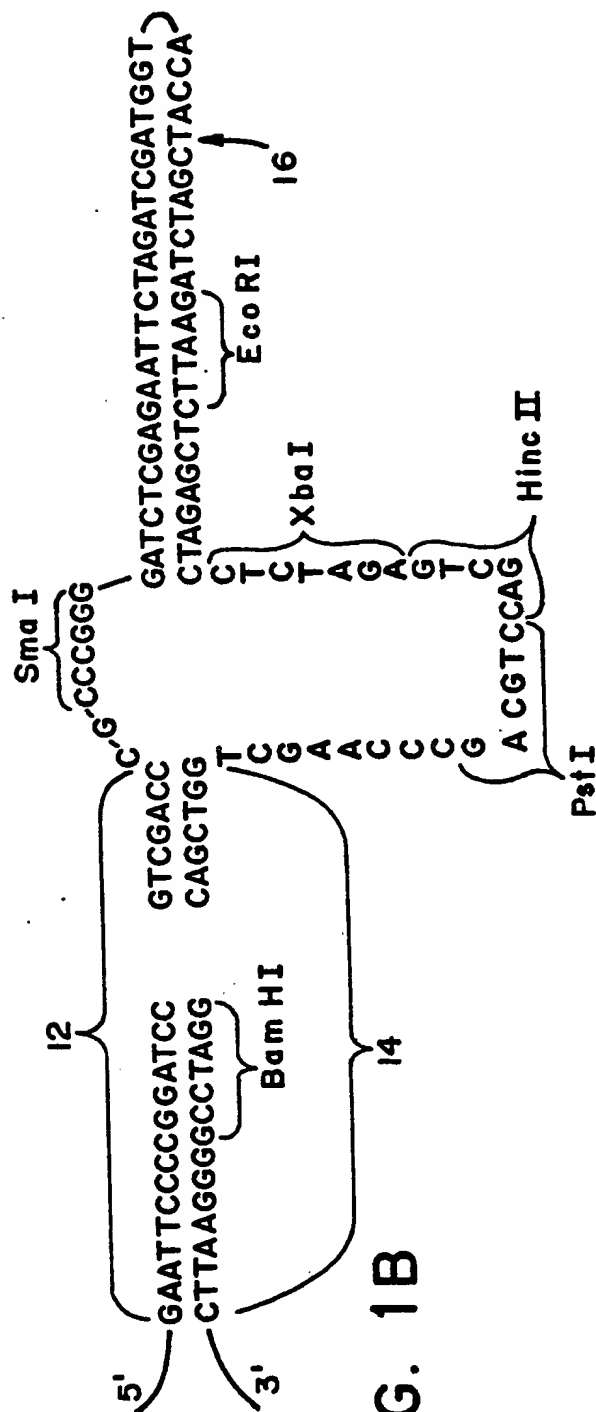


FIG. 1F





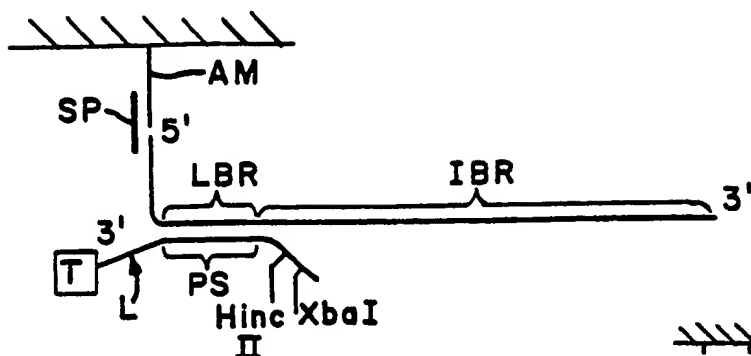


FIG.
1H

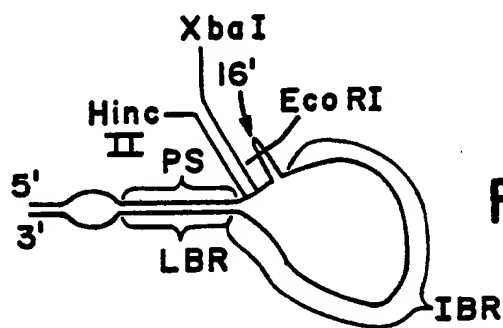
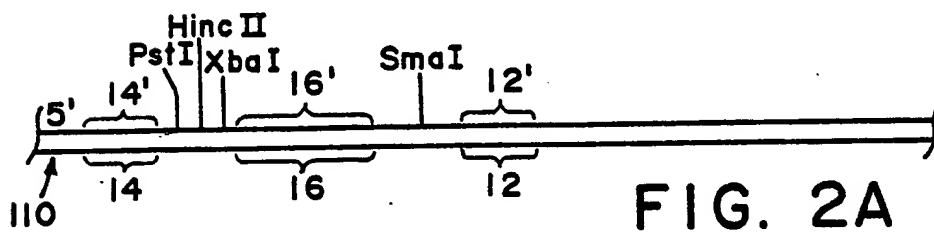
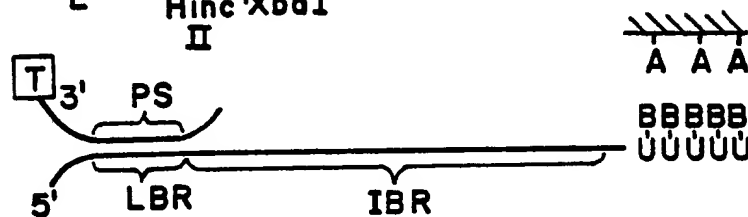


FIG. 2C

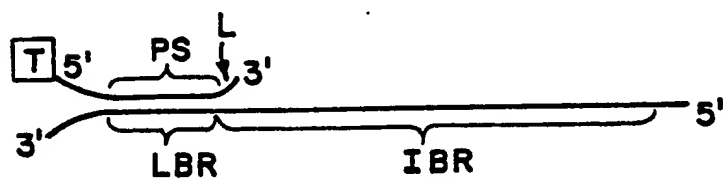


FIG. 2D

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FIG. 3B

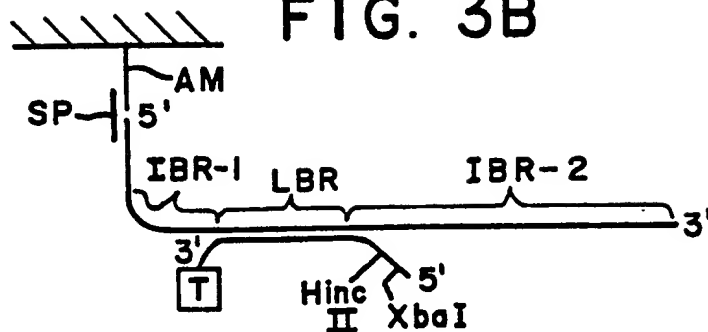


FIG. 3A

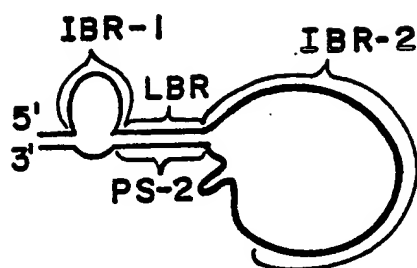


FIG. 3C

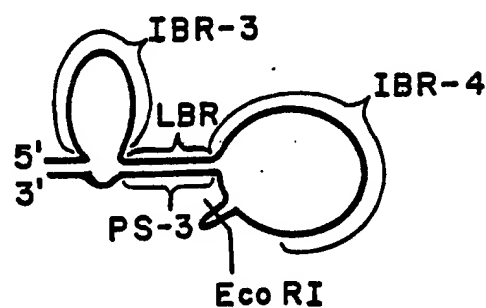


FIG. 3D

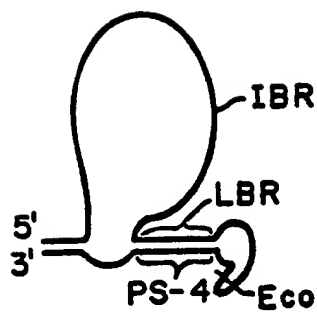
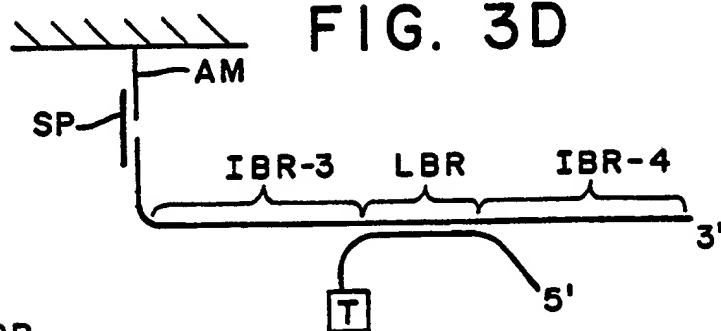


FIG. 3E

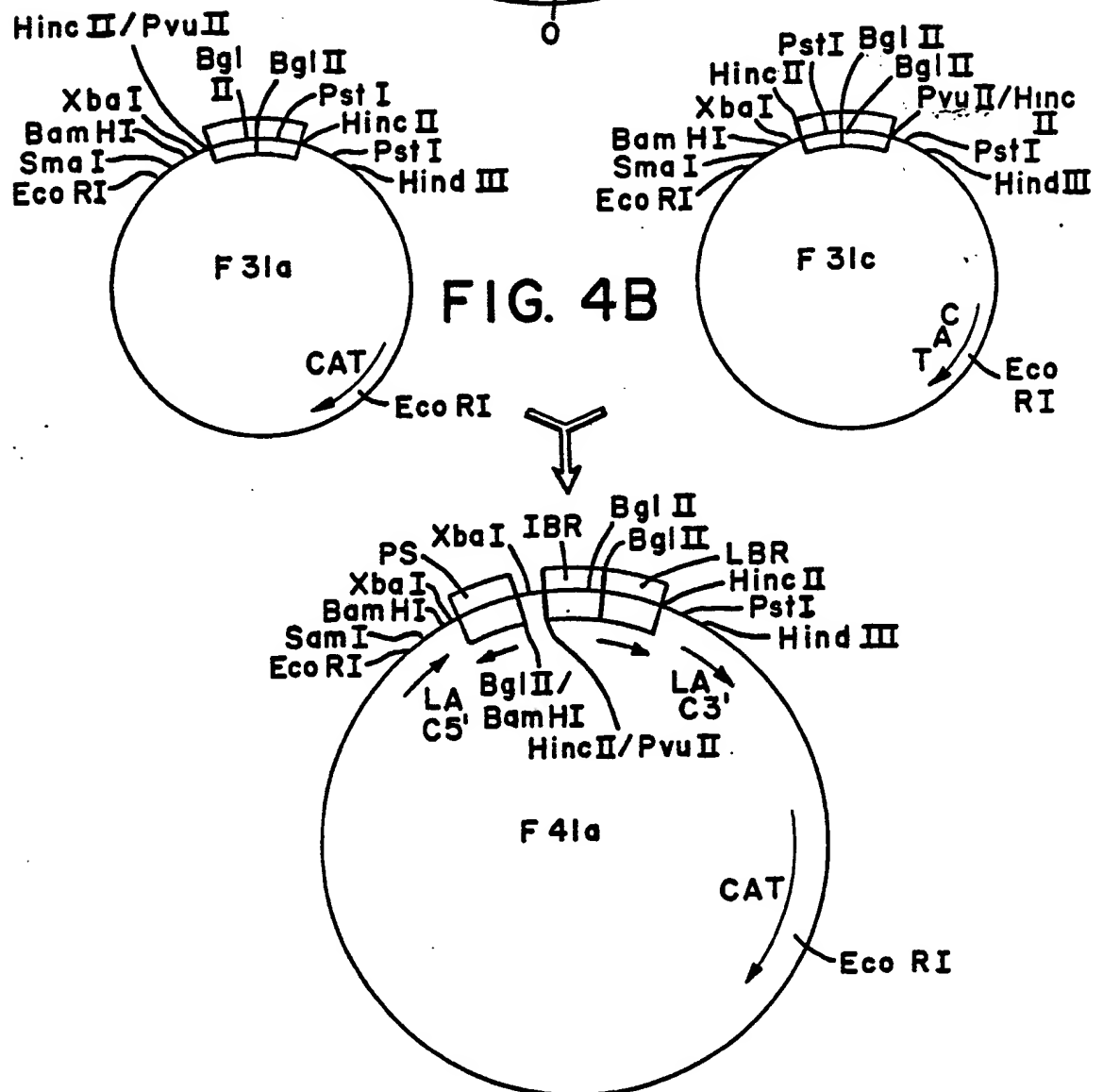
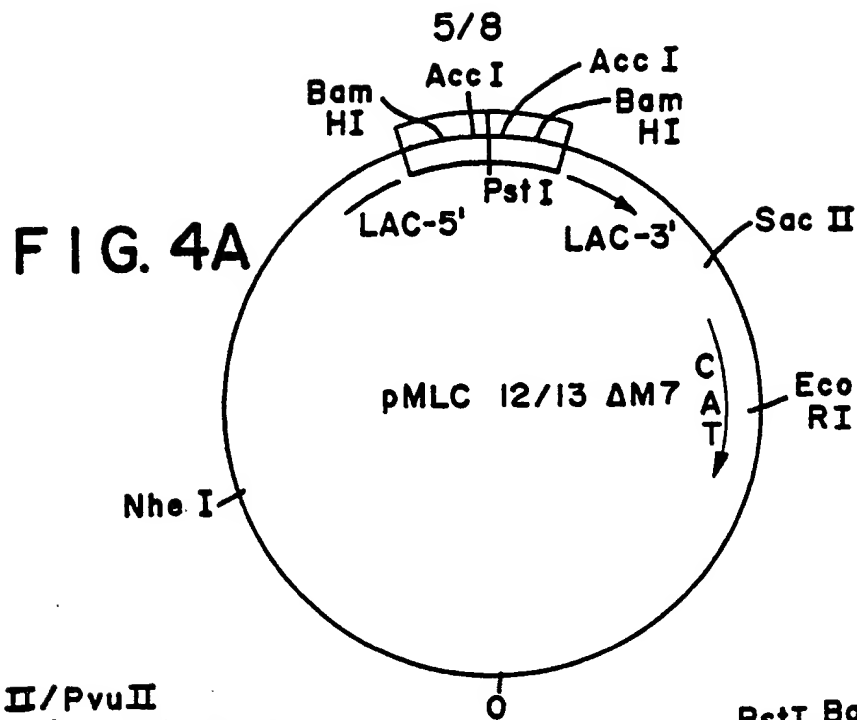




FIG. 4E

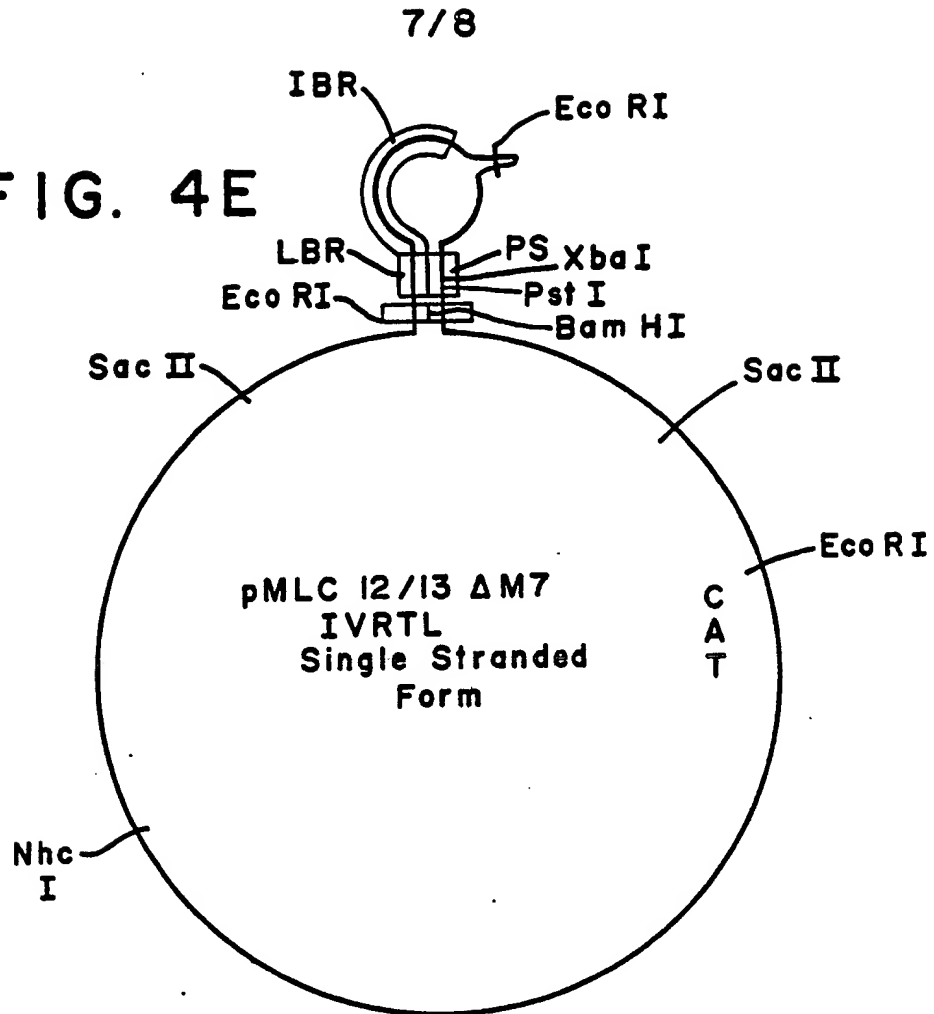
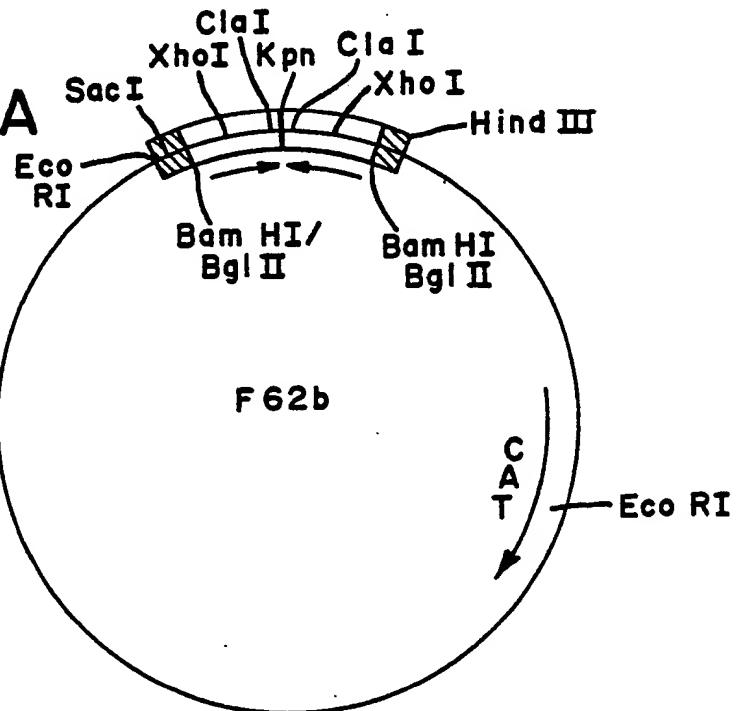
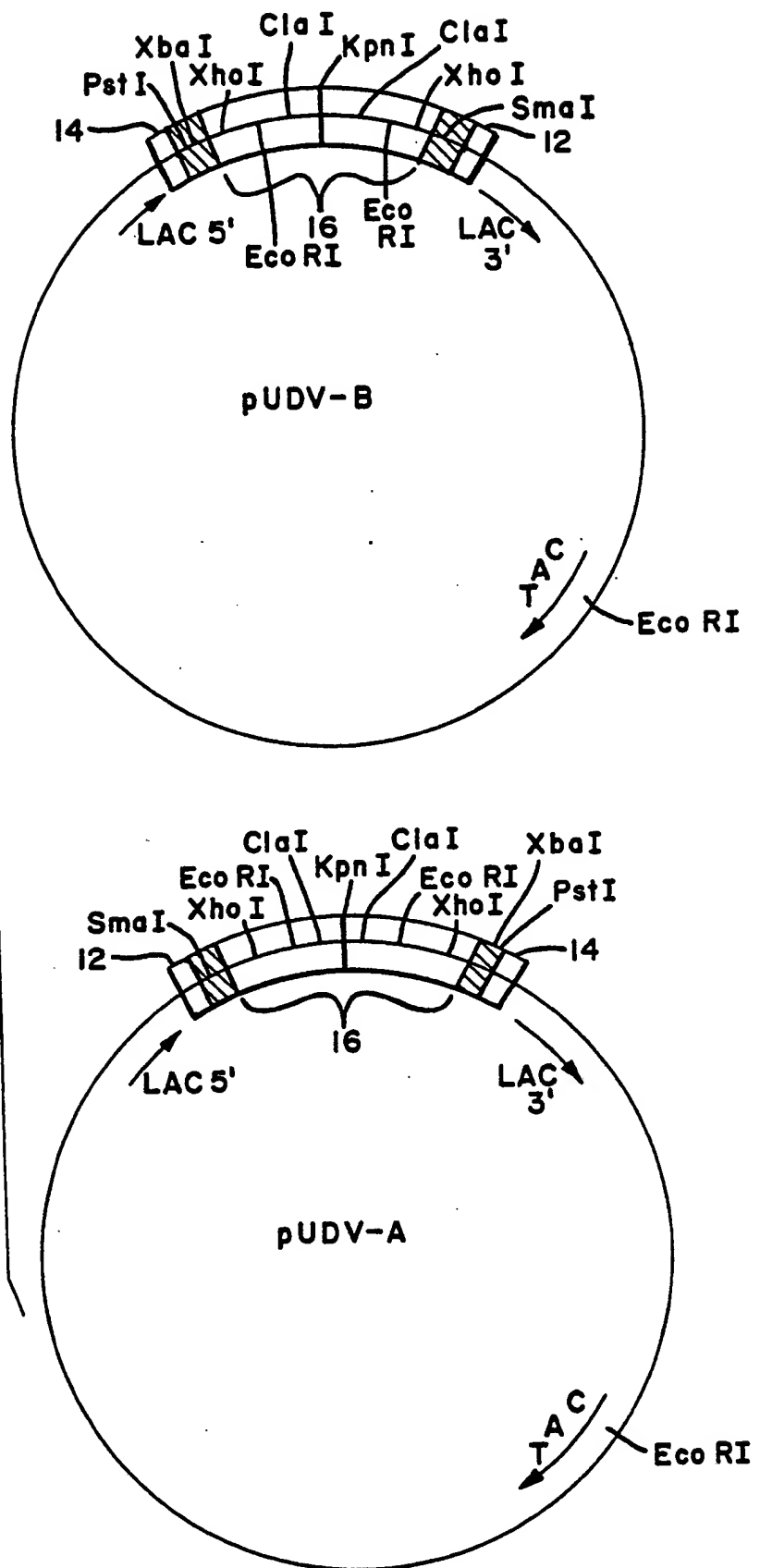


FIG. 5A



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FIG. 5B



INTERNATIONAL SEARCH REPORT

International Application No **PCT/US86/00742**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC INT. CL. ⁴ : C12Q 1/68; C12N 15/00; C07H 15/12 U.S. CL. : 435/6,172.3; 935/78; 536/27		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/6,91,172.3,320,803 436/501,94; 536/27 935/77,78	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	Nucleic Acids Research. Volume 9. No. 8, issued April 24, 1981, (C. GREEN ET AL.), "Reassociation rate limited displacement of DNA strands by branch migration", see page 1910	1-29
X Y	Methods in Enzymology. Volume 101. published 1983, by Academic Press (NY) (M.D. BEEN ET AL) "Cutting of M13mp7 Phage DNA and Excision of Cloned Single-Stranded Sequences by Restriction Endonucleases," see pages 90.91. 93 and 97.	1-12.25- 29 <hr style="width: 50%; margin: 0;"/> 13-24
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁶ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the International filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ¹		Date of Mailing of this International Search Report ²
07 June 1986		26 JUN 1986
International Searching Authority ¹		Signature of Authorizing Official ²⁰
ISA/US		Jeremy M. Jay

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
P, Y	WO, A, 8,504,674 (Life Technologies) 24 October 1985, see page 5, lines 6-13.	25-29
A	US, A, 4,321,365 (Wu) 23 March 1982, see column 2, lines 40-49	
Y	US, A, 4,358,535 (FALKOW) 09 November 1982, see column 1, lines 64-68. column 3, lines 35-48. column 4, lines 6.7 and 20.	1-29